Toward a Concept of Stretch Coupling in Smooth Muscle: A Thesis by Lars Thuneberg on Contractile Activity in Neonatal Interstitial Cells of Cajal

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Toward a Concept of Stretch Coupling in Smooth Muscle: A Thesis by Lars Thuneberg on Contractile Activity in Neonatal Interstitial Cells of Cajal

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ABSTRACT

The hypothesis was put forward by Thuneberg that rhythmically contracting interstitial cells of Cajal (ICC) were sensing stretch of the musculature and that this information was transmitted to smooth muscle cells via peg and socket contacts. The present study provides the evidence for the contractile nature of ICC as perceived by Thuneberg. The contractile activity is shown by video frame subtraction and by tracking areas of interest in sequential video frames. Thuneberg used neonatal ICC in culture maintained between two coverslips thereby allowing growth factors to quickly reach optimal concentrations. Contractions of ICC were seen to precede smooth muscle contractions. In addition, strong contractions were observed solely in branches of ICC. It is hoped that this communication will stimulate discussion about the contractile nature of ICC and that this phenomenon will eventually find its place amongst the physiological properties of the ICC networks of the gut musculature. Anat Rec, 293:1543–1552, 2010. © 2010 Wiley-Liss, Inc.

Key words: gut pacemakers; mechanotransduction; sensory mechanisms

In the 1970s, Lars Thuneberg's laboratory was working toward finding physiological hypotheses based on the pictures emerging from histochemical and electron microscopic data based on studies on the mouse and human intestine. It became clear to Thuneberg, Rumessen, and Mikkelsen that interstitial cells of Cajal (ICC) played an integral role in motility control of the gut and they became strongly convinced that the gut pacemaker activity resided in the networks of ICC. These years of research came to fruition in a dissertation by Thuneberg that was published in 1982 (Thuneberg, 1982) and a flurry of research papers with his coworkers that same year (Rumessen and Thuneberg, 1982; Thuneberg et al., 1982; Rumessen et al., 1982a,b). The pacemaker hypothesis proposed in these papers was strengthened by the fact that during those same years, Faussone Pellegrini in Italy independently had come to the same conclusion (Faussone-Pellegrini et al., 1977; Faussone Pellegrini and Cortesini, 1983). At the 9th International Symposium on Gastrointestinal Motility in Aix-en-Provence in September, 1983 (Thuneberg et al., 1984) and in later symposia (Thuneberg and Peters, 1987), Thuneberg showed videos of contracting ICC in culture. Now, 27

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years later, the pioneering work of Thuneberg has contributed to an ever increasing number of papers on the physiology and pathophysiology of ICC but the contractile properties of ICC have received little attention. All these years, Thuneberg remained fascinated by the phenomenon he could so easily observe in his cultures which he maintained between two microscope slides. In a letter to Jean-Pierre Timmermans as editor of the Anatomical Record, dated 6 May 2000, Lars Thuneberg wrote "*My plan is to follow up [his last paper* (Thuneberg and Peters, 2001)] *with a paper containing the evidence we have of spontaneous contractility and stretch sensitivity of ICC*." In his 2001 paper (Thuneberg and Peters, 2001), written just before memory loss occurred following a myocardial infarct, the hypothesis was pursued that pegs (transient club-like extensions of the smooth muscle cell membrane) are stretch sensors. It was noted that this "*organization makes sense if the ICC are spontaneously and rhythmically contractile cells, transferring a rhythmic, mechanical signal by stretching smooth muscle pegs buried in ICC sockets*." The present report is an attempt to write this article making use of Thuneberg's notes and figures left in his office, e-mail correspondence and further analysis of videos of contracting ICC recorded by Thuneberg. The objective of this series of experiments was to provide evidence for the hypothesis that spontaneous contractile activity is a key property of the myenteric network of pacemaker ICC.

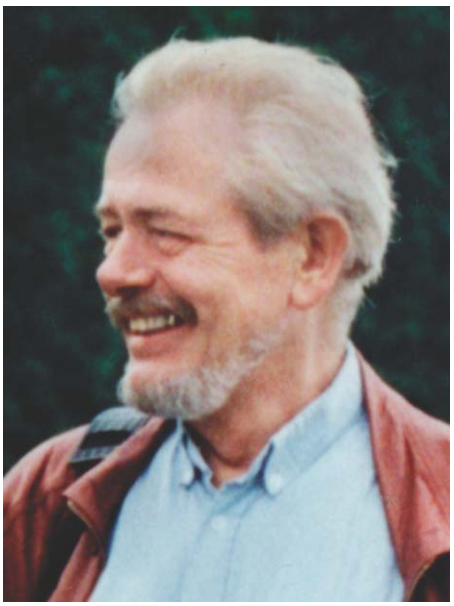

Fig. 1. Lars Thuneberg.

METHODS

All text within quotation marks and in italics were obtained from general notes, posters, figure legends, or e-mail correspondence written by Thuneberg. Figure 1 shows Lars Thuneberg in characteristic pose. At the moment of submission of this article (March, 2010), Thuneberg was living in downtown Copenhagen with little long-term or short-term memory.

The original material included VHS video recordings, which were converted into DVD format. From the DVD, 0.5- to 10-min segments were digitized into a digital camcorder (Sony DCR-TRV, 25 fps; 720 × 480 pixels) and several of these segments are loaded on hard disk (PowerBook G4; Apple) and converted to a digital movie (iMovie, Quicktime format). A program was developed to analyze the displacements of selected areas of interest (MotilityMap 2.0 written in RealBasic 2005). The approach of the algorithm is somewhat similar to what was described earlier (Lammers et al., 2001) and essentially consists of tracking a region of interest (ROI) in the movie frame by frame. In this case, however, instead of tracking an artificial marker (Lammers et al., 2001), the spontaneous movements of existing cellular structures or components were followed. The chosen approach was to select a ROI (Persson et al., 2003) and to search for the best matching region in the next frame. The search consisted of comparing the original ROI with regions of the same size in the next frame starting from the coordinates of the original ROI and searching in different directions. The comparison between the original ROI and each of the areas to be tested was performed by comparing the gray values of the pixels in the areas. To keep calculations and processing time to a minimum, gray values were expressed in integers (0–100) and the values of corresponding pixels were subtracted. All differences obtained from comparing two regions were then summed (sum-absolute-difference algorithm; Bohs et al., 1993; Bohs and Trahey, 1991). If an ROI is compared with itself, the differences between every pixel will be zero and the sum will also be zero. When this procedure is performed at the corresponding site in the next frame, then, in the absence of any movement, the differences will also be small but not zero due to small changes in alignment, lighting, and noise in the camera systems (Fig. 4D,E). If the ROI had moved significantly, however, then a search must be performed in the neighborhood of the original ROI to find the best match. As these movements may occur in any direction, the area has to be scanned in all directions. With trial and error, it was determined that a 9 × 9 search strategy was most reliable (Fig. 4C). Scanning smaller areas such as 7 × 7 or smaller often led to loss of the selected regions of interest, especially in the case of rapid cellular movements. The time step between every frame is 40 ms as determined by the capturing video camera (25 fps).

Explant Culture

Two- to four-days-old CD1 mice were killed by cervical dislocation and the gastrointestinal (GI) tract, starting from the stomach to the colon, was removed with intact mesenteric vascular bed to minimize stretch when transferred into a dissection dish, which was filled with M199 medium (Gibco). After removing the gut from the mesenteric vascular bed under a dissection microscopy, the jejunum (1.5-cm length) was obtained and mounted without stretch onto a Sylgard surface by insect pins (0.1 mm in diameter). The mucosa and submucosal were removed including the edge of the circular muscle that included the deep muscular plexus, leaving the outer circular muscle layer, the myenteric plexus, and the longitudinal muscle layer intact. The muscle strip was transferred into a Falcon Petri dish (VWR) with medium M199 and then cut into millimeter-size pieces. These

"explants" (5–10) were gently placed on collagen-coated (rat-tail collagen, Roche Diagnostics Corporation) glass coverslips in four well dishes (Nunc Serving Life Science) by using curved forceps, and immersed in M199 culture medium and incubated in 95% O_2 to 5% CO_2 at 37°C. The culture medium contained 10% fetal bovine serum (Gibco), 1% L-glutamine (Gibco), and 1% antibiotic–antimycotic (Gibco).

Immunohistochemistry

For staining antibodies of rat anti-ACK4 and rabbit anti-actin, the culture medium was removed from the coverslips, and all the cultured cells were fixed with cold acetone for 10–15 min. After rinsing, 5% normal goat serum (Vector Laboratory, Burlingame, CA) was added into each sample for incubation at room temperature for 30 min to reduce nonspecific staining. ACK4 antibody of 1:100 (Cedarlane, Hornby, ON, Canada) and 1:200 actin antibody (AnaSpec, SanJose, CA) were added followed by 12 hr at 4°C incubation. The secondary antibodies of Cy2 (anti-rat, Jackson ImmunoResearch Laboratory, West Grove, PA) and Cy3 (anti-rabbit, Jackson, ImmunoResearch Laboratory, West Grove, PA) were applied, 1:100 for 2 hr followed by phosphate buffered saline (PBS) washing for 3 × 5 min before being examined with a confocal microscope (LSM 510, Zeiss, Germany).

Electron Microscopy for Peg and Socket Assessment

We adopted a similar protocol as described in Thuneberg and Peters (2001). Briefly, freshly removed human ileum sections were rapidly immersed in osmic acid fixative for 1 min (2% osmic acid (Electron Microscopy Sciences) in 0.1 M phosphate buffer, pH 7.3), followed by addition of four volumes of aldehyde fixative (2% glutaraldehyde, 2% formaldehyde, 0.1% picrate, 0.1 M phosphate buffer, and final pH 7.3) at room temperature for 4–5 hr. Then tissues were transferred to fresh aldehyde fixative and stored overnight. On the second day, tissues were trimmed to 3-mm^3 pieces, postfixed in osmic acid fixative for 1 hr, dehydrated, infiltrated, and embedded with epon. Ultrathin sections were cut and grid stained with uranyl acetate and lead citrate. A Jeol 1200EX (Jeol Biosystem, Tokyo, Japan) electron microscope was used for examination and photography.

RESULTS

Contractions of ICC Assessed by Ultrastructure

Criteria used for assessing the contractile state of ICC in electron micrographs were the degree of surface folding and cytoplasmic density, however:

> *"Surface folding can only be applied if it is excluded that an observed folding pattern is due to passive deformation of a cell caused by active shortening of neighboring cells. The common close spatial connection between ICC and smooth muscle cells evokes speculation on whether observed surface irregularities are due to active or passive shortening of ICC. However, one location where cells with all the ultrastructural criteria of ICC may lie separated from muscle cells is the colonic submucosa" (Fig. 2). "Note that the contracted cell is an ultrastructural ICC, distinguished from fibroblasts by several criteria, not least the thick basal lamina."*[1]

Fig. 2. ICC fixed during a contractile state, contrasted by noncontracting fibroblasts. Dog colon submuscular plexus.

> *"By electron microscopy of random intestinal preparations, the longitudinal muscle shows variable degrees of contraction (except after diltiazem), whereas the circular muscle in most preparations shows little sign of contraction. When I obtained recordings from isolated segments of mouse small intestine it struck me that the longitudinal muscle nearly always was engaged in contractile activity, the circular muscle nearly always quiescent."*

> *"The ICC are dark in those locations where they are known to be preferentially oriented in parallel with the longitudinal axis of the gut: along primary fascicles (PF) of Auerbach's plexus, and when situated inside the longitudinal muscle layer. We postulate that the dark cytoplasm indicates a reaction to stretch, spreading from the actually stretched area to oral and aboral regions, probably caused by contractions spreading through the ICC-AP network (spontaneous contractions of ICC are prominent under cell culture conditions). We further postulate that – in the longitudinal muscle layer – the up to 50-fold increase in peg and socket junction numbers oral and aboral to radial or longitudinal stretch is mediated by the ICC network, this being well coupled by gap junctions. In support of this, peg and socket junction formation seems to occur in the order 1) between LM cells and ICC, 2) between LM cells in the layer next to ICC, 3) between LM cells further away. The schema was: Stretch evoked contractions of pacemaker cells → synchronization of pacemakers and deformation of stretch sensitive pegs → excitation of smooth muscle → phasic and/or tonic response".*

Video Analysis of Contracting ICC by Thuneberg

> *"I believe that I can demonstrate convincingly now, that the ICC are spontaneously contractile cells, also*

[1]All text within quotation marks and italics were obtained from general notes, posters, figure legends, or e-mail correspondence written by Thuneberg.

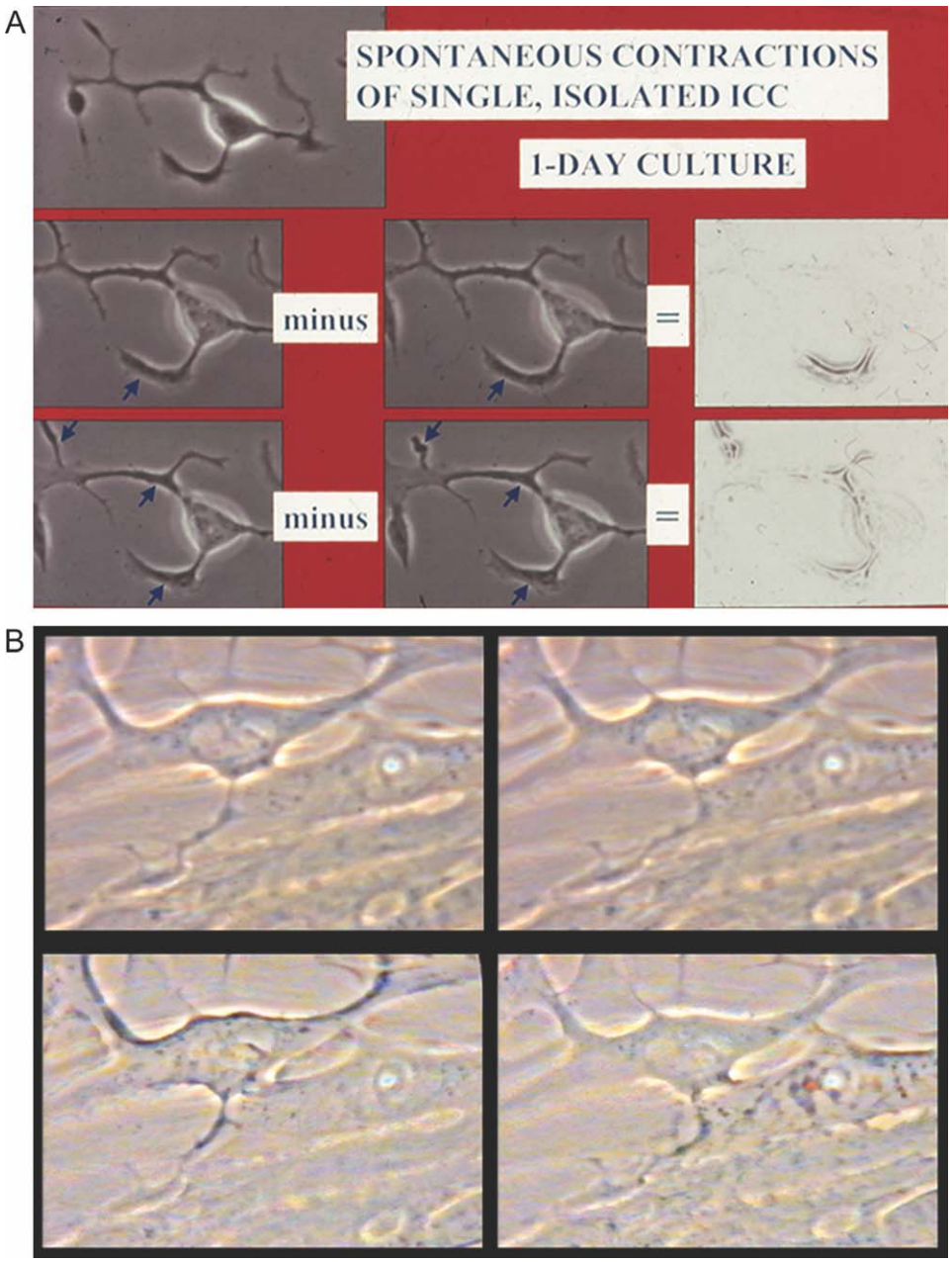

Fig. 3. **A**: Thuneberg captured contractile activity of ICC by subtraction of consecutive video frames. **B**: ICC contraction followed by smooth muscle contraction. One-day-old culture of ICC associated with an explant cultured at 37° in between two coverslips with a hollow ring of sylgard in between. A video camera was mounted on a phase contrast microscope to acquire the images.

when still in situ (in intact one-day explants and have one important other feature: that the cells are stretch-sensitive, also in one-day culture. The trick is to pick out video frames at regular intervals, a second or less, perform subtractions of subsequent images, which show nicely what is moving and the sequence of movements (Fig. 3). Both effects are impossible to demonstrate by comparison of just single consecutive frames, and the subtracted images demonstrate clearly those movements, which one has to see over and over again on the live video in order to grasp. Hence, the ICC are spontaneously and rhythmically contractile cells, also in vivo, and they are stretch-sensitive cells, like the smooth muscle cells. By their geometry with connected thin processes, the ICC would be expected to have lower thresholds to stretching forces than the smooth muscle cells and it is likely that stretch is a major factor in their coordination and frequency stabilization (Pollack, 1974).”

“The delay between ICC contraction and smooth muscle contraction, measured by optical means by analysis of video recordings of an established explant,

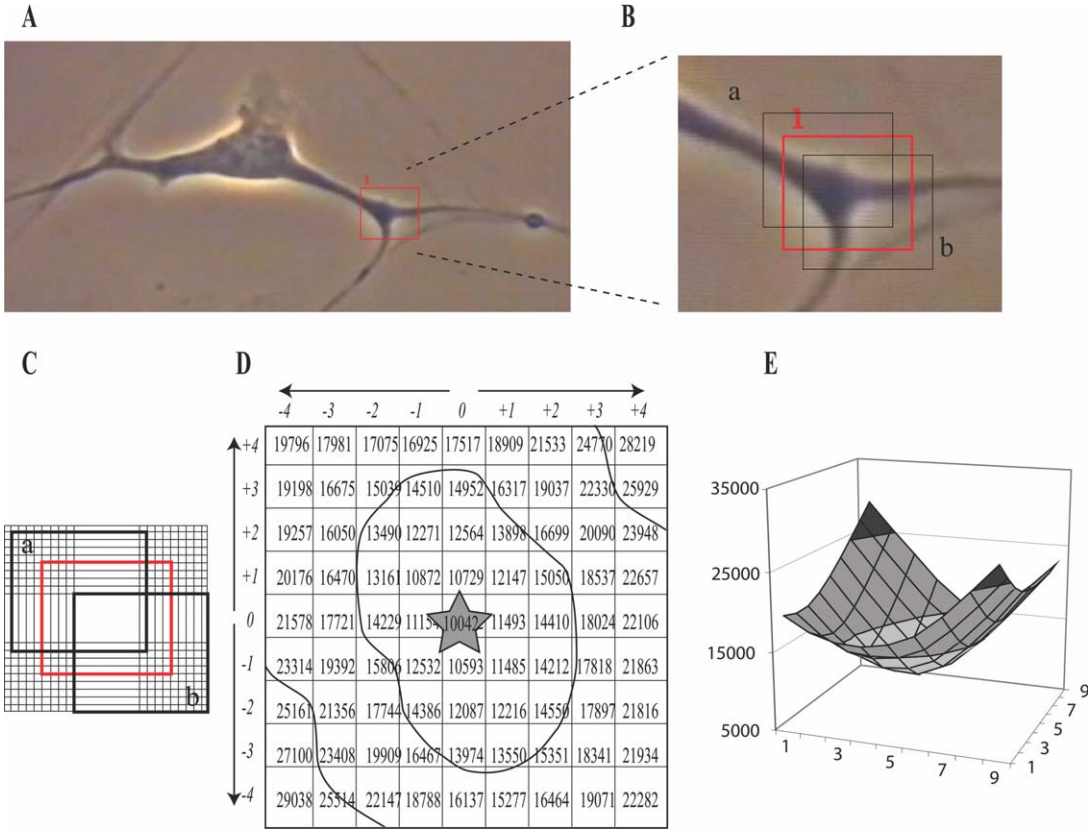

Fig. 4. Video snapshots and diagrams demonstrating the principles of tracking regions of interest (ROI). **A**: Depicts one frame of a video showing an ICC with several protruding processes. An ROI with a bifurcation in such a process was selected for tracking (red rectangle). The algorithm relates the gray values of the pixels in that ROI with their values in corresponding areas in the next video frame. The corresponding areas are scanned in all directions at 0–4 pixel distances from the center of the original ROI. Two such corresponding areas are shown in **B** and all the 81 tested areas are shown in **C**. **D**: Shows the results of calculating the similarities between the center ROI in this frame (shown in red in A–C) and the 81 regions in the next frame (low values mean high similarity; high values mean little similarity). As the cellular structure had moved very little between these two frames, the similarity was high in the center of the ROI and decreased considerably when areas located further away were evaluated. This relationship is also shown three-dimensionally in **E**.

contracting rhythmically at slow wave frequency, is close to 0.5 sec. When measured by video analysis in a 3–4 days culture of dispersed ICC/smooth muscle - on cell pairs consisting of an ICC with processes embracing a smooth muscle cell - the delay is more variable, but of the order of 1 sec. In both cases it is apparent that the ICC is the leading cell (meaning that there is a somewhat longer - approximately twice as long - interval between smooth muscle contraction and ICC contraction. The total cycle is of the order of 1.5 sec in mouse, frequency 40 cpm, which is close to normal slow wave frequency in mouse). Whether the ICC are always contracting is obviously a tough question. My observations on cultured ICC definitely support the constant rhythmic contractile activity, in contrast to smooth muscle under the same conditions. In aged cultured explants, when smooth muscle contraction has nearly vanished, the smooth muscle cells do appear to contract only when in contact with ICC, and the delay is as described above."

Computer-Assisted Analysis of Thuneberg's Videos

Figure 4 explains the analysis as outlined in the Methods section, and Fig. 5 elaborates on the contractile nature of the ICC that can also be observed at www.smoothmap.org (menu: Lars Thuneberg).

Figure 6A shows the movement of an ICC during a recording period of 34 sec. Three "regions of interest" had been selected in this cell. The spatial patterns of displacements are plotted superimposed on the first frame and in time below the frame. Spontaneous contractions occurred synchronously at all three locations with the displacements occurring toward the cell body. Figure 6B shows a similar behavior in another cell. Two peripheral components (#1 and #2) moved toward, and away from, the cell body (#3).

It was also possible with this technique to study spontaneous contractions in several ICCs coupled together. Two examples are shown in Fig. 7. In both cases, contraction in one cell would also induce displacements in other linked cells.

Peg and Socket Junctions in Cell to Cell Communication

The witnessing of contractile activity of ICC led Thuneberg to formulate the hypothesis put forward in this article on the relationship between contraction sensing

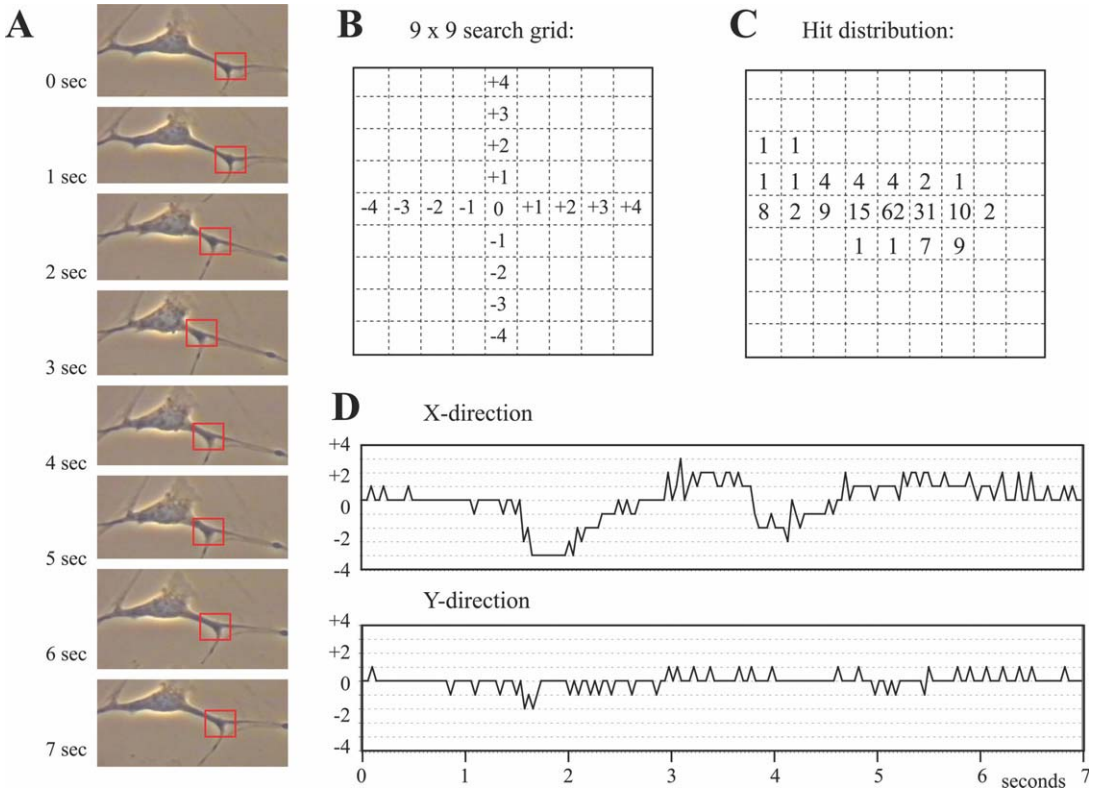

Fig. 5. Results of tracking an ROI for a period of 7 sec. **A**: Consecutive snapshots of an ICC and the ROI at 1-sec intervals showing the movement of the ROI toward the cell body and gradual return to starting conditions. **B**: Coordinates of the 9 × 9 search strategy. **C**: Number and location of highest similarities are found during this time period (comparable to the star in Fig. 4D). Most locations (N = 62) were found in the center of the successive ROIs indicating that during these time steps, the ROI did not move substantially. At other time steps, however, high similarities were found at other locations indicating that during these times steps, the ROI had moved. In this example, the displacements occurred predominantly horizontally and toward the left. This is also shown in **D** that plots the successive location of highest similarities. There was a strong displacement in Second 2 in the horizontal direction and a second weaker displacement in Second 4.

and peg and socket junctions. In a previous paper, the peg and socket junction characteristics were outlined and described as "*a distinct invagination of a cell process into a tightly fitting socket of a neighbouring cell*." The membrane structures "*were separated by a constant, narrow gap, not containing a basal lamina, or connective tissue components*." Thus, simple surface membrane folds should not be counted as peg-and-socket junctions. In close collaboration with Thuneberg and using his recommended specific fixation techniques, we set out to investigate the presence of peg and socket junctions in both the mouse and human small intestine. The specific fixation technique combined a rapid fixation of membranes (osmic acid) with an optimal preservation of intracellular protein (glutaraldehyde). The most remarkable advantage of this technique is the perfect fixation of membrane structures making the unique membrane to membrane aspects of the peg and socket junctions visible. The peg and socket junctions in the human small intestine were similar when compared with mouse tissues. In the small intestine of human, there are many more intramuscular ICC (ICC-IM) and ICC in septa (ICC-SEP) spread out in both circular and longitudinal muscle layers close to the myenteric plexus and appearing to be in continuity with the network of ICC-MP. Many peg-and-socket junctions were found between ICC-IM and smooth muscle cells (Fig. 8A), as well as between smooth muscle cells (Fig. 8B–D).

ICC in Explant Cultures Did Not Gain α-Actin

To investigate whether ICC differentiated into smooth muscle cells, α-actin was examined in ICC in cultures from day 4 to 21. As shown in Fig. 9, at day 7, both anti-c-kit positive cells (ICC) and anti-actin positive cells (smooth muscle cells) occurred without overlap. Similar results were obtained at day 21 (not shown).

DISCUSSION

The present study shows that interstitial cells of Cajal in short-term culture (24–72 hr after explanting a small piece of tissue in culture medium) exhibit marked contractile activity. Contraction occurs in several parts of the cell more or less independently or as contraction waves across the cell. It is likely that calcium rhythmically released from the sacroplasmic reticulum is responsible for the contractile activity. Intracellular calcium oscillations occur continuously and synchronously in ICC and smooth muscle cells (Yamazawa and Iino, 2002). These calcium oscillations are maintained in

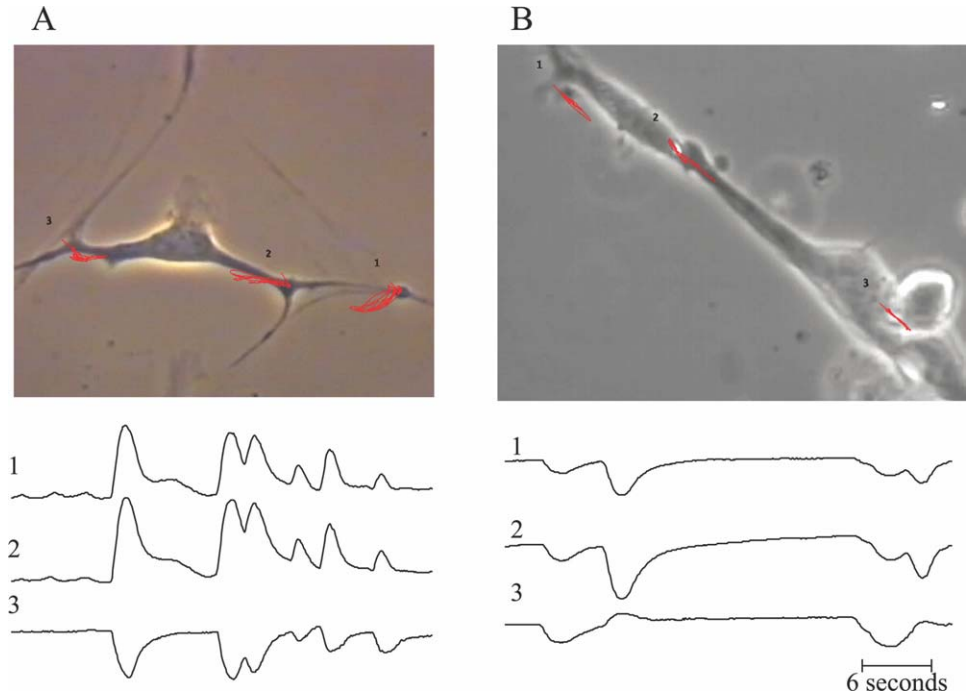

Fig. 6. **A** and **B** show two video frames of ICC with, in red, the displacements of three ROIs in each. Below each frame, the displacements of these three ROIs are also plotted in time. In A, the displacements of the three cellular components occurred in synchrony, moving to and from the cell body. A similar pattern was seen in B wherein two peripheral components (#1 and #2) moved in unison toward the cell body (#3).

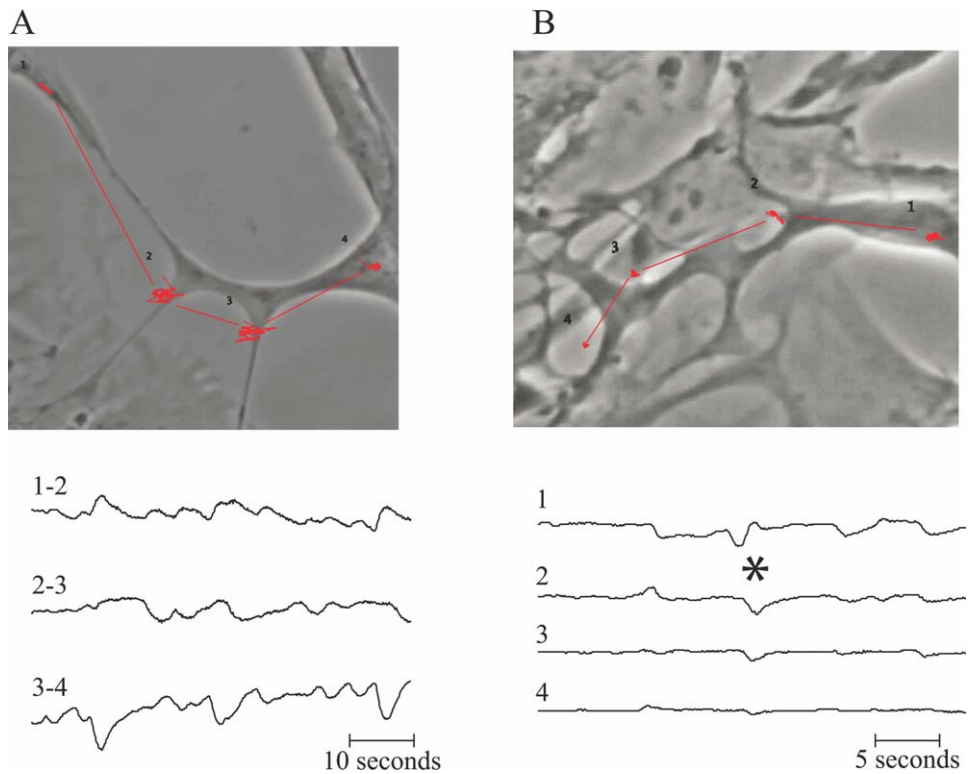

Fig. 7. Several ICC attached to each other are visible in the upper frames. In **A**, the three distances between the four adjacent cells were plotted in time, while in **B**, the absolute displacements were displayed. In both networks, contractions in one cell often affected movements in the other cells, although with decreasing amplitude (star in B traces).

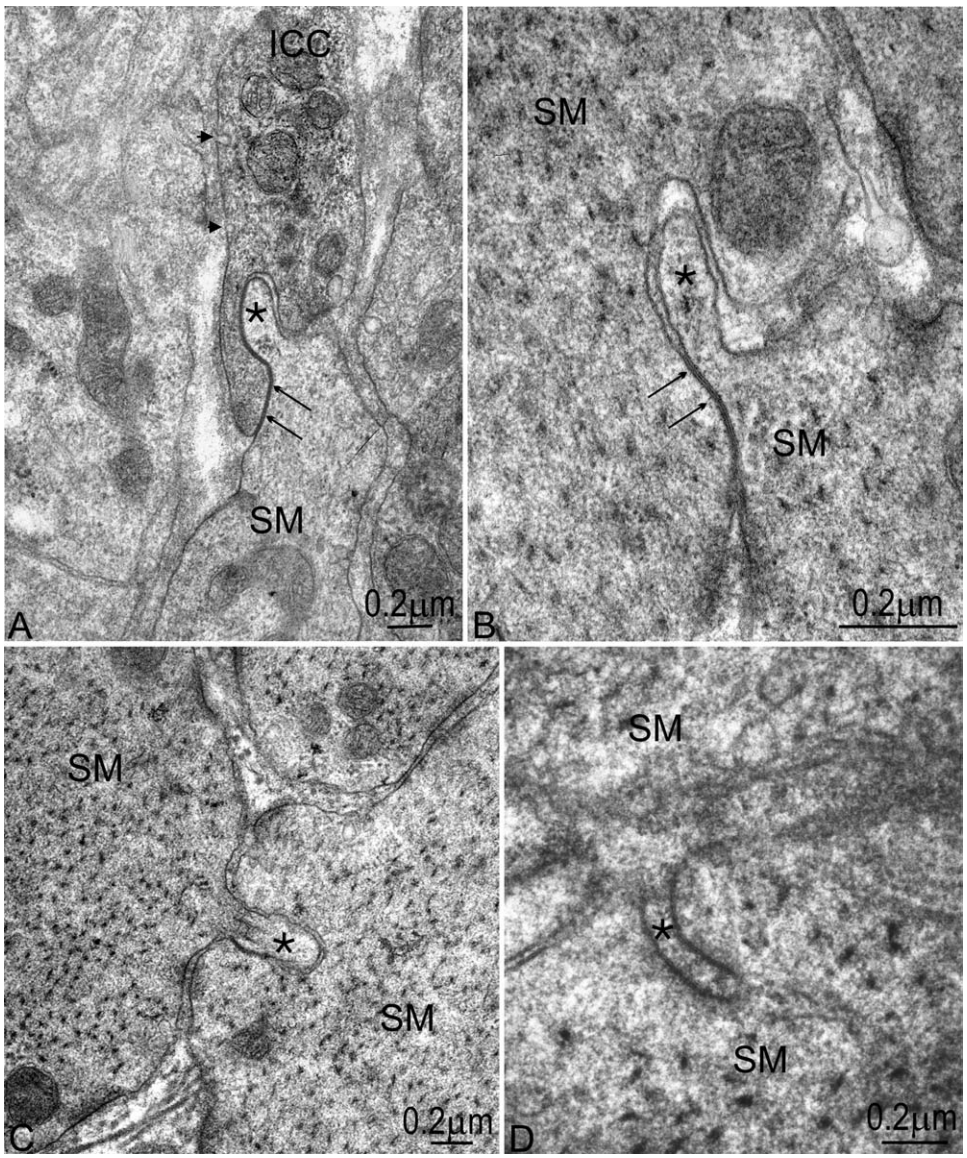

Fig. 8. Electron microscopy of peg-and-socket junctions (asterisks) in human ileum identified with primary osmic acid fixative. **A**: A peg-and-socket junction between an intramuscular ICC and smooth muscle cells (SM). **B–D**: Peg-and-socket junctions between adjacent smooth muscle cells (SM). In the areas of invaginations, membrane to membrane appositions were much closer than elsewhere. In A and B, peg-and-socket junctions were combined with gap junctions (small arrows) identified by the "three line structure" junction. Small arrowheads: caveolae.

the presence of L-type calcium channel blockers (Torihashi et al., 2002; Yamazawa and Iino, 2002).

The present study in conjunction with the data on peg and socket junctions (Thuneberg and Peters, 2001) suggests that "*a mechanical coupling exists between a contractile network of ICC and smooth muscle, the smooth muscle pegs buried in ICC sockets being responsible for the main coupling between the two. With some speculation, the model would include a highly stretch-sensitive network of ICC, perhaps even synchronized by a mechanism involving contractile responses to stretch, and including the one-way mechanical coupling with smooth muscle. The same basic mechanism was suggested in a brilliant paper by Pollack (1974) to explain the coupling and impulse delay in the atrioventricular (AV) node of the heart. The new development is that there is a morphological basis in the peg-and-socket junctions (and these are prominent also in the AV node).*"

> *"It is my - educated - impression, that the stimulation of peg and socket junction formation starts upon stretch of the ICC-AP or ICC-DMP (Thuneberg and Peters, 2001) (and they would be more likely to sense the rinsing of the intestine, than the smooth muscle cells, with few pegs at the time). It is indeed very likely that peg and socket junction formation starts with the ICC inducing increased numbers of contacts with their neighbor muscle cells (of LM only). It may well take further action*

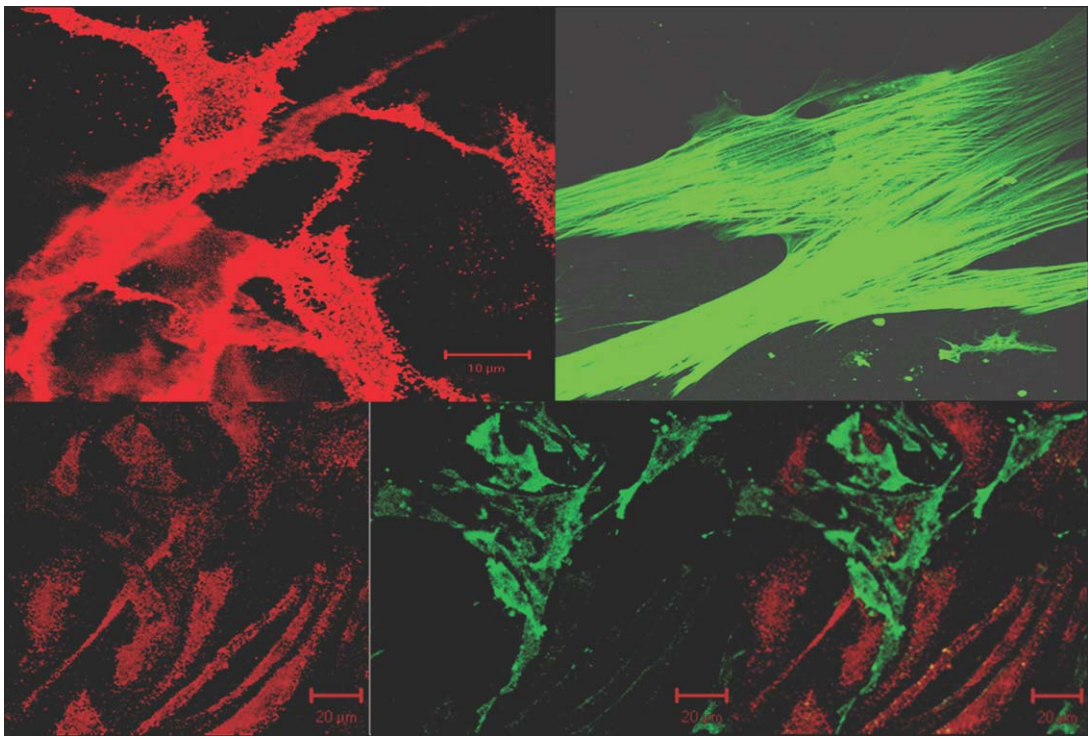

Fig. 9. Staining with actin and c-kit antibodies in day 5 explant culture. Upper panel: c-kit (red), actin (green); × 63 objectives. Lower panel: c-kit (green), actin (red); × 20 objectives.

(more force and/or prolonged stretch) to stimulate the muscle cells into forming peg and socket junctions between them. It is significant, that within LM, there is a gradient in numbers of peg and socket junctions with numbers increasing towards the ICC-AP. In the CM, it is the ICC-DMP which seem to lead the battle, with what appears to be a higher threshold for induction of peg and socket junctions. Different thresholds (ICC<LM<CM) make lots of sense, if you consider the ICC the alarm-clocks, and the work-load on the longitudinal muscle being more related to moving along and mixing the more fluid surface contents, while the hard work of the circular muscle is called upon, when it has to produce occluding contractions against a semi-solid content, as in segmentation motility!

The notion that ICC may be stretch sensors has been proposed by others as well (Gabella, 1979; Rumessen et al., 1982; Fox et al., 2000; Won et al., 2005). That distention may be followed by peg and socket junctions has been explained in the previous paper (Thuneberg and Peters, 2001). There is no evidence that formation of peg and socket junctions is quickly followed by their dissolution. Hence, distention-induced peg and sockets may have special relevance to longstanding changes in distention and/or muscle tone.

The proposal that a contractile network of ICC senses muscle stretch is novel but the notion that ICC can contract will be controversial, given the fact that myosin is not detected by electron microscopy (EM) as thick filaments (Wang et al., 2002) and that myosin is not detected by reverse transcription polymerase chain reaction (RT-PCR) (Epperson et al., 2000). The contraction is therefore likely not mediated by Type II myosin that forms thick filaments (Eddinger and Meer, 1997) but by another as of yet unknown type of myosin. Clearly, the molecular nature of the contractile activity needs further study. Under certain culture conditions, ICC may de-differentiate into smooth muscle–like cells. This is probably not relevant to most studies mentioned here since ICC moving out of explants can be visualized 24 hr after culture, hence, too short to have acquired smooth muscle actin and/or smooth muscle myosin by differentiation in culture media. In addition, the present study shows that ICC obtained by explant culture do not gain actin for at least 3 weeks. However, concerns about culture-induced contractile proteins may apply to some comments made on aged cultures. It is possible that observations of non-contractile isolated ICC obtained by chemical dissociation is due to alterations in ICC by the dissociation process. The culture conditions exploited by Thuneberg are special in that the culture takes place in a very small volume in between two glass coverslips. This may be the critical factor for the fact that isolated ICC can be observed to contract 24 hr after the initiation of the explant: the concentration of essential growth factors is apparently optimal under these conditions. Recent studies on calcium imaging *in situ* in adult mice show networks of ICC rhythmically gaining intense increases in intracellular calcium (Torihashi et al., 2002; Park et al., 2006). This is generally not associated with contractile activity in the network. Hence, the characteristics and roles of contractile activity in neonatal and adult ICC networks have to be further explored.

ACKNOWLEDGMENTS

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