Involvement of Interstitial Cells of Cajal in Pacemaker Activity of Canine Colon

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Introduction

Previous studies have shown that electrical slow waves of the colon originate from a discrete population of cells along the submucosal surface of the circular muscle layer and conduct in a non-regenerative manner into the depth of the circular muscle (Smith, Reed & Sanders, 1989). The submucosal surface of the circular layer is lined with interstitial cells of Cajal (ICC; Berezin, Huizinga & Daniel, 1988), and it has been suggested that these cells serve a pacemaker function in the gut (Faussone-Pellegrini, 1983; Li, 1940; Suzuki, Prosser & Dahms, 1986; Thuneberg, 1982). Most of the evidence for this hypothesis has come from morphological studies, since physiological roles of ICC have been difficult to determine. This is because: i) ICC are small and networks of ICC are thin; both are difficult to identify in living tissues (Suzuki et al., 1986); ii) Specific, vital markers for ICC that would allow identification in living tissues have not been developed; iii) ICC form networks of cells that are electrically coupled to each other and to adjacent smooth muscle cells; recording from ICC with conventional microelectrodes cannot determine whether electrical events recorded in ICC originate in these cells or in smooth muscle cells; iv) Chemicals used to lesion ICC may also have pharmacological effects on smooth muscle cells (Sanders, Burke & Stevens, 1989).

Our laboratories have combined a number of morphological, electrophysiological, and fluorescence techniques to study the role of ICC in generating rhythmicity. This paper briefly reviews some of the findings from these studies.

Methods and Results

Morphological association between ICC and pacemaker activity

Slow waves are an important electrical activity in colonic circular muscle and appear to be the primary event linking excitation with contraction (Christensen, Caprilli & Lund, 1968; Sanders & Smith, 1986; Barajas-Lopez & Huizinga, 1989). We have found that L-type Ca$^{2+}$ channels are activated and not fully inactivated at the level of depolarization reached during slow waves (Langton, Burke & Sanders, 1989). Thus, sustained entry of Ca$^{2+}$ through these channels during slow waves appears to be the crucial step for excitation-contraction coupling in these muscles.
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The amplitude of slow waves is greatest along the submucosal surface of the circular layer, and dual impalements of 2 cells (i.e., one at the submucosal surface and another within the depth of the circular layer) show a delay between activation of the submucosal cell and a cell in the middle of the circular layer (Smith et al., 1987). We also found that removal of a band of cells along the submucosal surface abolished slow wave activity from the bulk of the circular layer, but slow waves could still be recorded from cells within the excised strip (Smith et al., 1987). These findings suggest that slow waves originate from a population of cells along the submucosal surface of the circular layer. We, and others (Berezin et al., 1988), have examined the morphology of this "pacemaker" region and found 3 cell types present in abundance (Fig. 1): (i) smooth muscle cells, (ii) ICC, and (iii) neurons. ICC form an electrical syncytium, a few cells thick, that lines the submucosal surface of the circular muscle. ICC are abundantly electrically coupled to each other and, less frequently, to neighboring smooth muscle cells. Neural processes are also abundant, and varicose regions of these processes often appear in close proximity to ICC.

Upon examination of cross sections of the circular layer (i.e., sections cut transverse to the long axis of the circular fibers), one notices that the circular muscle is divided into bundles (Ward & Sanders, 1990). The submucosal surface invaginates, creating septa, which divide the circular layer (Fig. 2a). We have found that ICC also populate these septal structures and form a similar structural relationship with neighboring smooth muscle cells. This suggests that the slow wave pacemaker region may also extend into the septa. We tested this by removing a thin strip of muscle from the submucosal surface along septal and non-septal regions (Ward & Sanders, 1990). We found that slow wave activity was abolished from the non-septal regions, confirming our earlier observation (Smith et al., 1987), but slow wave activity could still be recorded near the septa (Fig. 3). Besides slow wave activity, there are not obvious differences between smooth muscle cells of septal and non-septal regions (i.e., they have similar resting potentials, similar morphology, etc.). It is their proximity to ICC that is the major difference between muscle cells of the 2 regions. Thus, these data further support the notion that ICC generate pacemaker activity.

Our data also suggest that ICC are involved in regenerative propagation of slow waves. We have reported that slow waves conduct in a non-regenerative manner through the thickness of the circular layer (Smith et al., 1987). But in tissues and in vivo, slow waves propagate longitudinally and circumferentially without decrement for distances inconsistent with non-regenerative propagation (Sanders, Stevens, Burke & Ward, 1990; Christensen & Hauser, 1971A & B). Therefore, a regenerative pathway must exist for slow wave propagation. We found that removal of a thin band of cells along the submucosal surface disrupts longitudinal and circumferential regenerative slow wave propagation and the decay of slow waves with distance was similar to the decay in these events through the thickness of the circular layer (Smith et al., 1987; Sanders et al., 1990). We also found that slow waves propagate along the septal surfaces in a regenerative manner (Ward & Sanders, 1990). Therefore, in canine colon, the bundles of circular fibers appear to be wrapped in a pacemaker/regenerative surface. Electrical excitability and coupling of ICC may be the essential elements of this pacemaker/regenerative surface, and the properties of ICC that allow them to be pacemakers may also...
Fig. 1. Electron micrograph of pacemaker region along submucosal surface of circular muscle layer of canine colon. Smooth muscle cells (CM) are lined with interstitial cells (ICC). These are characterized by clusters of mitochondria (M), a basal lamina (BL), and caveolae (not clearly shown at this magnification). Also present along submucosal surface are nerve bundles (NB). ICC form frequent gap junctions with each other (GJ) and with adjacent smooth muscle cells (Modified from Ward et al., 1990).
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Fig. 2. Top panel shows photograph of an unstained, cross-sectional preparation under low power magnification with bright field illumination. The muscularis externae was cut transverse to the long axis of the circular muscle. The preparation was pinned in cross-section exposing submucosa (SM), circular muscle (CM) and longitudinal muscle (LM) layers. The submucosal surface is optically dense and invaginates frequently into the circular layer, forming septa (S), which divide the circular layer into fiber bundles. Bottom panel shows similar cross-sectional muscle strip stained with rhodamine 123. Note fluorescent band along submucosal surface of circular muscle layer and staining of septa. Electron microscopy showed that this fluorescence was due to uptake of dye into ICC and neural elements. Smooth muscle cells were not significantly labeled with the dye.
allow them to actively propagate slow waves. Indeed ICC networks may be the normal
pathway for active propagation in gastrointestinal muscles.

Excitability and spontaneous activity of ICC

As noted in the Introduction, it is not easy to demonstrate whether ICC originate rhythmic
activity or even whether they are excitable cells using intact muscle preparations. Therefore,
we have attempted to address these questions by developing a preparation of isolated ICC. We
have isolated the submucosal pacemaker region by dissection and enzymatically dispersed cells
from this region (for details see Langton, Ward, Carl, Norell & Sanders, 1989). These
dispersions yielded a mixed population of cells. Most were smooth muscle cells, but another
cell-type with prominent nuclear regions, dark granular cytoplasm, and multiple processes was
also observed (Fig. 4). Since these are the gross features of ICC, further studies were perfor-
med to compare the ultrastructure of these ICC-like cells with ICC in situ. Cells identified
under phase contrast were marked, and then fixed and embedded in resin. After relocating
these cells, they were sectioned and examined with transmission electron microscopy. The
Fig. 4. Isolated ICC shown with Hoffman contrast optics. Note multiple processes and prominent nuclear region.

ICC-like cells were found to have many of the ultrastructural features of ICC: i) abundance of mitochondria, ii) cisternae of endoplasmic reticulum, iii) pronounced ovoid nuclei with a sparse heterochromatin, iv) abundance of thin filaments, and v) caveolae along the plasma membrane.

Isolation of ICC provided an opportunity to study the electrophysiology of ICC. Cells were studied with the patch clamp technique using whole-cell and off-cell patch recording configurations. Under whole-cell conditions, depolarization from holding potentials between -80 and -50 elicited transient inward and sustained outward currents (Langton et al., 1989b; Fig. 5). Expression of these currents provided the first concrete evidence that ICC are excitable cells.

Blockade of outward currents by dialysis with pipette solutions containing 50 mM Cs⁺ and inclusion of TEA (40 mM) in the bath solution, allowed characterization of the inward current component. The inward current reached a peak within 5-10 ms of depolarization and then decayed to a sustained level that persisted for the duration of test potentials. Peak current averaged about 300 pA. The inward current was blocked by nifedipine, suggesting that it was primarily due to Ca²⁺ flux through “L-type” channels.
A portion of the outward current was Ca\textsuperscript{2+}-dependent. The channels responsible for this current were studied in excised patches. The voltage- and Ca\textsuperscript{2+}-dependence of these channels was studied by measuring current responses to potentials ramped from +100 to -50 mV at Ca\textsuperscript{2+} concentrations ranging from 0.1 to 0.5 μM. As described elsewhere (Carl & Sanders, 1989), the open probability of channels as a function of voltage and Ca\textsuperscript{2+} concentration was determined from averaged current responses. Increasing Ca\textsuperscript{2+} shifted the activation curve to more negative levels, suggesting that these channels may become more active as Ca\textsuperscript{2+} enters cells during excitable events.

Since voltage-dependent channels survive enzymatic dispersion, it is possible that the mechanism responsible for rhythmicity also remains intact. This was tested under current clamp conditions. From resting potential, or when small holding currents were applied to polarize cells to about -80 mV, spontaneous depolarizations were observed. These events were
characterized by a relatively rapid upstroke to about -20 mV, partial repolarization to a plateau level, and then, after several seconds, repolarization to the resting level (Fig. 6). These events were very similar to the slow waves recorded from intact muscles.

Spontaneous activity was further tested by loading isolated ICC with fluorescent probes sensitive to voltage or intracellular Ca^{2+}. These cells demonstrated rhythmic oscillations in voltage and intracellular Ca^{2+}. Although individual cells oscillated at a relatively constant frequency, the range in frequency between cells was from 0.1 to 7 cycles per min.

**Can ICC serve as pacemakers in situ?**

Despite the evidence that ICC are excitable cells and spontaneously generate rhythmic activity (i.e. pacemaker activity), the question remains whether these cells are actually capable of pacing the syncytium of smooth muscle cells they are electrically coupled to in situ. The problems of a point source of current activating a 3-dimensional syncytium of smooth muscle cells are well documented (cf. Holeman, Neild & Lang, 1990). We measured up to 500 pA of inward current from single ICC at 23°C (Langton et al., 1989b), and we would expect this current to increase by 2-3 fold at 37°C (Ward & Sanders, unpublished observations). Even this magnitude of current may not be enough to elicit depolarization in coupled smooth muscle cells, since injection of this amount of current with a microelectrode produces little or no voltage response (Publicover & Sanders, unpublished observations). In situ ICC do not appear to function in isolation; they are organized into an electrical syncytium. And this is likely to be an essential feature of electrical rhythmicity. One hypothesis might be that pacemaker activity is regenerated and amplified within the ICC network and this current enters the smooth muscle syncytium through multiple points of contact. Since the ICC network is a surface lining the circular layer, activation is accomplished by a 2-dimensional current source. This reduces the amount of current required of individual ICC and greatly improves the safety factor for propagation between ICC network and smooth muscle. Unfortunately this will be a very difficult hypothesis to test rigorously since it would demand: i) vital identification and differentiation of ICC and smooth muscle cells; ii) simultaneous intracellular recording from multiple sites; and iii) a highly specific means of regulating gap junction conductance, preferably the junctions specifically between ICC and smooth muscle cells. Another method to test the feasibility of the ICC network hypothesis will be numerical models, but this will require precise information about the electrical and morphological properties of both cell-types.

**Use of rhodamine 123 to label and lesion ICC function**

Another means to test the hypothesis that ICC serve as pacemakers is to develop a technique to specifically lesion these cells. If loss of ICC results in a loss of rhythmicity, then one might deduce that either ICC generate this activity or that they play a vital role in transmission. Whatever technique is used, it should be quantitative (i.e. removing all ICC) and specific (i.e. affecting ICC but not smooth muscle). It is highly unlikely that dissection techniques will be suitable for removing ICC. For example, in the colon the septal ICC are inaccessible to dissection. Thuneberg and colleagues have used methylene blue as a chemical means to lesion ICC (Thuneberg, Johanson, Rumenssen & Anderson, 1983), but we have found
that this agent has non-specific effects on smooth muscle cells (Sanders, Burke & Stevens, 1989). It would be extremely useful to develop more specific labels for ICC and to find compounds that are specifically toxic to ICC.

Since ICC contain an abundance of mitochondria, we have tested the fluorescent dye rhodamine 123 (Ward, Burke & Sanders, 1990), a specific mitochondrial marker (Johnson et al. 1980), for its ability to specifically label ICC vs. smooth muscle cells. Rhodamine 123 has cytotoxic effects on some tumor cells (Nadakavukaren et al., 1985; Modica-Napolitano & Aprille, 1987), suggesting that uptake of this compound might be a means to specifically lesion labeled cells. Therefore, we have also studied the effects of rhodamine 123 on the slow wave activity of intact muscles.

Cross sectional strips of muscles, exposing the entire muscularis externa, were incubated with rhodamine 123 (26 μM) in a 95%-5% O₂-CO₂ incubator at 37°C for 15 minutes. After incubation, the muscles were washed with oxygenated Krebs solution for an hour. Then the muscles were fixed, sectioned, and examined with light and electron microscopy. Under fluorescence microscopy at 485 nm, a thin band of intense labeling was observed along the submucosal border and extending down into the septal structures. Close examination revealed that individual cells were labeled with the dye (Fig. 2b). Ultrathin sections were cut from the same blocks and examined with transmission electron microscopy. These studies revealed that rhodamine 123 labeled both ICC and neural process. The labeling was selective for ICC vs. smooth muscle.

Electrophysiological experiments were performed on intact muscle preparations to determine whether conditions which resulted in selective uptake of rhodamine 123 affected slow wave activity. Rhodamine 123 (26 μM) caused disruption of slow wave activity in the 10 preparations studied. This effect was characterized either by dysrhythmias (i.e. sporadic slow wave activity) or total abolition of rhythmic activity. It is unlikely that this effect was due to toxic effects of rhodamine 123 on nerves, since slow wave activity persists in the presence of nerve toxins and after cold storage of muscles (Sanders & Smith, 1986). Patch clamp experiments were also performed on isolated smooth muscle cells to determine whether rhodamine 123 affects background conductance or voltage-dependent currents of these cells. These studies suggest that the conditions and concentration of rhodamine 123 used to label ICC have little effect on smooth muscle cells. In general, studies with rhodamine 123 suggest that this compound may be useful to quickly indicate the location of ICC in heterogenous tissues, but this histological technique must be checked with EM since cross-labeling of enteric neurons occurs. The effects of rhodamine 123 on slow wave activity further suggest a pacemaker role for ICC.

**Cultured ICC as a model of electrical rhythmicity**

Despite techniques to isolate ICC, large scale experimentation, using a variety of modern techniques, awaits a means to either purify preparations of freshly dispersed cells or culture these cells. Recently, we have been engaged in efforts to develop a tissue culture model of ICC. This will facilitate electrophysiological studies, allow application of biochemical and imaging technologies, and facilitate collection of enough cellular material to allow development of specific antibodies. Of course, many of these aspirations depend upon retention of the ICC
Enzymatic dispersions of ICC were plated in low density. Individual cells having the morphology of ICC were identified with phase contrast microscopy and isolated from other cells with teflon rings. These cells were cultured through several divisions. The clonal cells within the ring were isolated and sub-cultured. Cells of several passages from several of these cell lines were used in electrophysiological experiments and in studies in which intracellular Ca\(^{2+}\) was studied with the fluorescent indicators Fluo 3 or Indo-1. These studies have shown that cultured ICC retain their rhythmic capability and electrical changes are accompanied by oscillations in cytosolic Ca\(^{2+}\). This preparation is likely to be a very valuable tool in studying the origin and regulation of electrical rhythmicity in gastrointestinal muscles.

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References


Li, P. (1940). The intramural nervous system of the small intestine with special reference to the innervation of the inner subdivision of its circular muscle. *J. Anatomy (Lond.)* **74**: 348-357.


