A FUNCTIONAL ANALYSIS OF GASTROINTESTINAL MOTILITY IN THE GUINEA PIG AND HUMAN

A thesis submitted in total fulfilment of the requirements of the degree of doctor of philosophy

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August 2012

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 Summary of findings

The work in this thesis highlights the involvement of both neurogenic and myogenic mechanisms in the generation and coordination of gastrointestinal motility. The literature review of chapter 1 covered the broad range of elements that have an involvement in gastrointestinal motility. Many of the studies reviewed in chapter 1 were based on analysis of the electrical and/ or mechanical activities of smooth muscle, with input from neurogenic and myogenic sources. In many of these studies an interesting physiological process would have occurred during their experimentation. After the initial setup of these preparations, but prior to the commencement of recordings, a period of 'warming up' or 'equilibration' is usually required for smooth muscle preparations: before this time has elapsed they may appear completely inactive. With time, the responses of these tissues return as preparations recover from the apparent trauma caused during setting up. Often such recovery periods are mentioned in the methods section of papers, however this phenomena has not been previously studied. There are almost no papers that consider the mechanistic causes for this loss of responses (Brookes, 1988). However this period of unresponsiveness demonstrates that there are powerful intrinsic mechanisms in these tissues, which can apparently 'turn off' their normal physiological functions. Much of this thesis aimed to characterise the changes to gastrointestinal physiology that may account for this loss of responses, and to understand the mechanistic causes for this response.

In chapter 2, I characterised a number of physiological changes to smooth muscle cells over this period. Intracellular electrophysiology demonstrated that within the 30 minutes of preparation setup, circular smooth muscle cells from the ileum and colon had: absent or small junction potentials, hyperpolarised resting membrane potential, high input resistance and little dye coupling. Mechanical recordings from similar preparations lacked tone and failed to generate spontaneous contractions. Responses

significantly changed over 120 minutes following dissection: the amplitude of junction potentials increased, resting membrane potentials depolarised, input resistance decreased, dye coupling increased, basal tone increased and the amplitude of spontaneous contractions increased. Release of neurotransmitter from neurons were probably unaffected since large fast excitatory post synaptic potentials were recorded from neurons impaled within the first 30 minutes following dissection. This suggests that changes in smooth muscle physiology were largely responsible for the loss of responses following preparation setup. It also suggested that the associated reduction of gap junction coupling may contribute to the loss of responses in smooth muscle cells.

This theory was tested in chapter 3, where pharmacological blocking of gap junctions in responsive preparations, reduced dye coupling and increased input resistance, significantly reduced the amplitude of junction potentials and reduced the amplitude of spontaneous contractions. Fast IJPs are mediated by purines such as ATP. To test whether circular smooth muscle cells were able to respond to neurotransmitters immediately following dissection, exogenous ATP was applied directly onto the impaled cell. The amplitude of hyperpolarisations evoked by exogenous ATP for cells impaled within 30 minutes of dissection was negligible compared to cells impaled after 120 minutes. This supported the suggestion that smooth muscle physiology was the primary site affected by preparation setup, and this led to the suppression neuromuscular responses. The amplitude of sIJPs and EJPs were also reduced following dissection, or by gap junction blockers. It was unfortunate that application of exogenous mediators for these junction potentials did not successfully evoke a response in 'equilibrated' smooth muscle cells. This is an area that should be pursued further in the future.

In chapter 4 I determined which particular elements of setting up activated mechanisms that contributed to the loss of responses and associated loss of coupling. A second, transient reduction in responses (measured by fIJP amplitude) and reduction in dye coupling was caused by re-cutting the edges of responsive

preparations. These changes occurred several cell lengths from the site of damage suggesting damage is activating mechanisms that affect the tissue, not just at the stie of damage. Transient reductions in temperature and over-stretching responsive preparations did not replicate the unresponsive period. Negligible fIJP amplitudes after setup persisted in preparations dissected in modified Krebs solution with either: limited Ca^{2+} concentration; indomethacin to limit prostaglandin synthesis; or ketotifen to stabilise mast cells. This suggests that these elements are unlikely to be mediating the loss of response following preparation setup. In preparations where damage was limited by keeping the mucosa intact, the amplitude of fIJPs was initially reduced, however not to the negligible levels as seen with completely dissected preparations. These results indicate that damage from cutting preparations and removing the mucosa suppresses gap junction coupling and smooth muscle cell responses. The mediator or mechanism activated and/or released by damage was not identified in these studies.

In chapter 5 I investigated the neurogenic and myogenic mechanisms that contribute to motor patterns recorded in the human colon. There is some discrepancy between the types of contractions recorded in the intact human colon *in vivo* versus *in vitro* recordings from narrow segments of tissue. I tested whether the frequency of contractions recorded from narrow segments of human colon *in vitro* differed from larger segments, however I found no significant differences. Spontaneous contractions, termed slow phasic contractions, occurred at less than 1 cycle per minute. This was consistent with recordings by Huizinga and colleagues (Huizinga et al., 1988, Huizinga et al., 1985). In chapter 5 hexamethonium and TTX reduced the frequency of contractions compared to control frequencies, but not their amplitudes. This suggests these myogenic contractions are an all or nothing event and that neural input, which may involve cholinergic neurons, can modulate the timing of the contractions. In chapter 1 I highlighted that functional studies on neuro-neuronal transmission in human colon were lacking. Evidence from labelling studies indicated that circular muscle motorneurons extend up to 10mm (Wattchow et al., 1997, Wattchow et al., 1995). This information was used in chapter 5 to design a preparation where the role of cholinergic interneurons in generating ascending

contractions could be assessed. Electrical stimulation applied more than 10 mm aboral to the recording site generated an ascending contraction. The amplitude of evoked contractions was not significantly different from the previously mentioned spontaneous slow phasic contractions, and the evoked contractions reset the timing interval of these contractions. This suggests that a myogenic pattern generator mediates the slow phasic contractions at less than 1 cycle per minute. However, activation of ascending neural pathways can trigger a premature contraction and reset the spontaneous cycle. Electrical stimulation continued to evoke contractions in the presence of hexamethonium, indicating that cholinergic interneuronal transmission was not required for the ascending neural input.

6.2 Other considerations

6.2.1 Post dissection loss of responses- not just gap junction coupling

Results from chapters 2 and 3 indicate that the loss of gap junction coupling following preparation setup may account for much of the loss of responsiveness after dissection. However, in most preparations smooth muscle still had some, residual limited dye coupling yet junction potentials were usually unmeasurable. When gap junction blockers were applied to responsive preparations, dye coupling was nearly entirely blocked yet the amplitude of junction potentials was only reduced. Therefore, reduction of gap junction coupling is probably not the only mechanism, which causes the loss of responses; another mechanism must also contribute. Damage, which I have shown to contribute to the loss of both coupling and responses, releases a multitude of mediators, which could modify cellular functioning in ways that may be independent of effects on gap junctions. In a similar study on warm-mediated change in responses from smooth muscle cells of the coronary artery, the responses of preparations stored in refrigerated Krebs solution for 24 hours were compared with freshly dissected vessels not stored in cold solution (Keef and Ross, 1986). The resting membrane potential of smooth muscle cells from 'stored vessels' was around -40mV in cells impaled within 20 minutes of setting up, hyperpolairsed to -70mV after 30 to 60 minutes, then depolarised to -50mV after 240-360 minutes (Keef and Ross, 1986). Exposure to ouabain, Na^+ pump blocker, for 5 minutes during the 30-60 minute interval depolarised cells from around -75mv to -

40mV. Keef and Ross suggest that this hyperpolarisation was due to activation of the electrogenic sodium pump triggered by temperature changes. Enzymes and metabolic processes do not function as well at cold temperatures, this would reduce the production of ATP and therefore limit the activity of the sodium pump. However ions are still able to passively cross cellular membranes, shifting the electrochemical gradient. At physiological temperatures, over-activation of the pump to restore the electrochemical balance may account for the hyperpolarisation. When vessels had fully equilibrated, and presumably when ionic gradients were restored to normal, the pump switched off and the hyperpolarisation was reduced.

6.2.2 Gap junction coupling and neuromuscular responses: the role of coupling between smooth muscle cells, ICC and fibroblast like cells

Gap junction uncoupling of smooth muscle, by either pharmacological intervention or following preparation setup, resulted in reduced post junctional responses and diminished hyperpolarisations to exogenous ATP. It was postulated that uncoupling of smooth muscle cells to an intermediary cell such as the ICC or fibroblast-like cells, may account for this loss of responses. However of all dye fills of several hundred circular muscle cells not one was dye coupled to either an identifiable ICC or a fibroblast-like cell. Therefore, while it can be concluded that gap junction coupling between smooth muscle modulated neuromuscular activity in this preparation, the involvement of an intermediary cell remains speculative. Repeating these experiments in the longitudinal muscle layers may prove to be useful. In chapter 2, the validity of using input resistance and dye coupling as measures of gap junction coupling were tested by comparing the values of responsive longitudinal muscle cells versus circular muscle cells. There is little evidence for gap junction coupling in the longitudinal muscle layer (Daniel and Wang, 1999, Gabella and Blundell, 1981, Mikkelsen et al., 1993), although with a long enough dye filling protocol, longitudinal muscle cells in the canine colon are dye coupled (Farraway et al., 1995). Evidence from the murine gastric corpus indicates that the ICC-IM in the longitudinal muscle layer are closely aligned with cholinergic and nitrergic nerve fibres (Song et al., 2005). It would be worthwhile testing either the recovery of post junctional responses in the longitudinal muscle layer after dissection, or the responses of these cells after recovery and with gap junction blockers. If coupling of

the smooth muscle cells to each other is important for normal neuromuscular function then responses should be unaffected in these cells. Alternatively if coupling to an intermediary cell is required then responses would be expected to follow similar trends to the circular muscle layer.

In chapter 3 responses to exogenous ATP were sensitive to gap junction blockade. This was a key finding for supporting the hypothesis that neuromuscular transmission may require an intermediary cell, and there is evidence that Fibroblast-Like Cells may mediate this response (Kurahashi et al., 2011). It would be informative to test whether an agent known to act only on smooth muscle cells during the equilibration period and in gap junction blockers. The problem with this is that it is not certain whether any such agent exists. Using RT-PCR and immunohistochemistery ICC have been shown to express receptors for many transmitters including 5-HT, acetylcholine, VIP, substance P, and ATP (Epperson et al., 2000, Wouters et al., 2007, Chen et al., 2006). Acetylcholine and NO act via ICC-IM (Burns et al., 1996, Ward et al., 2000a, Suzuki et al., 2003a); it is possible that transmitters assumed to act directly on smooth muscle cells also require an intermediary cell. It is also not clear whether function of intermediary cells is affected by dissection, or are all effects solely due to gap junction uncoupling. To settle these issues recordings from intermediary cells need to be carried out. This may require high resolution optics and/or live labelling of the cells, for example with antibodies to surface markers or GFP linked to cell specific promoters.

6.2.3 Release of neurotransmitter is not affected by preparation setup

In chapter 2, fast excitatory post synaptic potentials were recorded from *S* neurons impaled within the first 30 minutes of dissection. This suggests that the release of excitatory neurotransmitters from nerve terminals was not impeded following dissection. While this provides an indication that the mechanisms of neurotransmitter release are not globally affected in the preparation, it is not definitive evidence that the mechanisms of ATP release from inhibitory motorneurons are not impaired. ATP bioluminescence assays may be a potential technique to measure the release of ATP

from motorneurons after different time points following dissection. This technique has been used in this preparation (Ambache et al., 1986); more recent advances may make it more sensitive to the levels of ATP release from neuron terminals. However, consideration would have to be taken into account for the release of ATP from interneurons.

6.2.4 2-APB blocks gap junctions and IP3 receptors

In chapter 3, 2-APB at 50μM was used to block gap junctions. In responsive circular smooth muscle cells this significantly reduced fIJP amplitude, reduced ATP evoked hyperpolarisations and reduced dye coupling. RMP had a trend towards hyperpolarisation and input resistance increased. However, 2-APB is also used to inhibit IP_3-dependent Ca^{2+} release, which has a role in slow wave generation. Gastric smooth muscle cells from mutant mice which lack IP_3 type 1 receptors fail to generate slow waves (Suzuki et al., 2000). However, some of these results using 2-APB as an IP_3 receptor inhibitor may also be explained by the action of this agent on gap junctions. For example in the guinea pig antrum at 60μM 2-APB inhibited slow waves recorded from smooth muscle cells (Hirst and Edwards, 2001), and at 50μM the propagation of Ca^{2+} waves amongst circular smooth muscle cells was also inhibited (Hennig, 2004). The latter effects may have been partly due to inhibition of gap junction coupling.

6.2.5 Is the lack of responses following preparation setup specific to the guinea pig?

The lack of responses following preparation setup occured in both the guinea pig ileum and colon, and responses were inhibited by gap junction blockers in both preparations. However, whether this phenomenon is specific to the guinea pig, or occurs in to other models, is yet to be tested. Certainly, in my mechanical recordings from the human colon in chapter 5, spontaneous contractions were negligible at the start of recordings then recovered with time. This was quite similar too the recordings in the guinea pig colon from chapter 2. This apparent loss and recovery of responses has been noted in the human colon elsewhere (Duthie and Kirk, 1978). Although mechanical recordings do not necessarily reflect gap junction coupling,

they may prove useful in documenting the recovery periods of other specimens to see if they compare with the guinea pig.

6.2.6 The loss of responses is "just damage"

The results from chapter 2, 3 and 4 highlight that damage is a contributor to the loss of responses and coupling of smooth muscle cells. The significance of these results could be underestimated, as damage is sometimes considered "unphysiological". However, what the present study highlights is that there are intrinsic mechanisms within the gut that can modulate gap junction coupling and thereby affect motility. Damage from dissection can activate these mechanisms, but this does not mean that activation is only caused by damage; other more physiological factors could potentially target these mechanisms. For example, increases in intracellular Ca^{2+} caused by damage could activate protein kinase C. It is also established that neurotransmitters and modulators can also activate PKC, opening the possibility that gap junction coupling could be modulated during normal physiological conditions. This remains to be tested empirically.

6.2.7 Ascending pathways in the human colon: cholinergic and tachykinergic

In chapter 5, electrical stimulation of ascending interneuronal pathways in the human colon resulted in an oral contraction. These evoked contractions persisted in the presence of hexamethonium indicating that cholinergic neurons are not required for neuro-neuronal transmission in the human colon. Therefore one or more other excitatory neurotransmitters must be involved. Immunohistochemical studies suggest there may be two classes of ascending excitatory interneurons in the human colon: the majority are immunoreactive for cholineacetyl transferase (ChAT) but some are not (Porter et al., 1996, Porter et al., 2002b). This differs from the guinea pig ileum where all ascending interneurons are immunoreactive for ChAT (Costa et al., 1996). Interneurons in the human colon are also immunoreactive for tachykinins (Wattchow et al., 1997), therefore it seems likely that this class of neurotransmitters maybe involved in ascending excitatory neurotransmission. There is some suggestion that tachykinins have more of a role in gastrointestinal motility in human colon compared

to animal studies (Cao, 2006, Cao, 2000). The experimental design used in chapter 5 provides the ideal layout to test this. The length of specimens used was long enough for interneronal pathways to be activated and the pharmacological barrier permitted superfusion/ direct application of different pharmacological agents to either segment. At present, a comparison of the immunoreactivity of myenteric neurons for ChAT and TK has yet to be done.

6.2.8 An alternative pattern generator in the colon

In chapter 1 and results from chapter 5, evidence was presented for a myogenic pattern generator oscillating at a slower frequency than that of slow waves recorded in animal studies (Huizinga et al., 1988, Huizinga et al., 1985). This pattern generator is likely to be located within the myenteric region of the colon wall (Rae et al., 1998). However, mechanical patterns occurring at a similar frequency are not recorded by colonic manometry (Dinning et al., 2010b). Therefore, either *in vivo* recording techniques lack the resolution to detect this pattern or this pattern does not present as a manometric event in *in vivo studies.* High resolution colonic manometry is likely to become more commonplace in *in vivo* studies in the future; perhaps discrepancies between *in vitro* and *in vivo* recordings will be resolved as technologies improve. However, it should also be considered that this slow phasic pattern may be more pronounced *in vitro,* due to experimental artifact. Similar contractions in the pig colon, were blocked by TTX, but reoccurred when preparations were stretched (Huizinga et al., 1983). Therefore it may be that when pinning and applying a small amount of stretch to preparations in this experimental setup, an ample amount of stretch was applied to activate the motor pattern. Removing the specimen from the body eliminates the influence of extrinsic nerves, hormones, blood-borne factors and so on. However, this does not mean that the activity pattern does not occur *in vivo*, it may be that this pattern generator paces the activity of other events but is not large enough to evoke contractions *in vivo.* Slow phasic contractions may normally be inhibited by ongoing enteric neural activity that is disrupted in preparations *in vitro*. At this stage there is little that can be done to control for the different conditions produced by *in vitro* recordings.

6.3 Conclusion

This thesis has investigated the roles of some myogenic and neurogenic mechanisms that contribute to gastrointestinal motility in the intestines. Modulating gap junction coupling between smooth muscle cells influences their responses to neuromuscular transmission. Intrinsic mechanisms, triggered by damage from cutting preparations and removing the mucosa can trigger these mechanisms. This represents a powerful mechanism that is capable of modulating gastrointestinal motility. The activity of myogenic pattern generators in the human colon can be modulated by intrinsic inputs. Activation of ascending interneuronal pathways can stimulate this pattern generator to alter the timing of contractions. These ascending neural pathways do not require cholinergic transmission between neurons to be functional. This thesis has demonstrated the existence of several novel mechanisms that may contribute to the control of contractility of the mammalian gut. Identifying their functional significance will be a potentially important challenge.