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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4. UNDERSTANDING THE MECHANISMS THAT LEAD TO GAP JUNCTION UNCOUPLING AND ASSOCIATED UNRESPONSIVENESS FOLLOWING DISSECTION OF THE GUINEA PIG ILEUM

4.1 Introduction

Following dissection of smooth muscle preparations there is a period of reduced responsiveness. In chapters 2 and 3 I demonstrated that following dissection of the guinea pig ileum and colon, a loss of junction potentials is associated with reduced gap junction coupling. As responses recovered over the ensuing 120 minutes, gap junction coupling increased. Pharmacological inhibition of gap junction coupling in preparations that had achieved full responsiveness diminished junction potentials. The question remains as to which of the elements of dissection cause the loss of responses and what mechanisms do these elements activate to cause gap junction uncoupling. Since neither the effects of dissection nor physiological modulators of gap junction coupling between gastrointestinal smooth muscle cells have been previously tested, a range of possibilities were considered in this study.

In my previous studies, preparations were dissected in Krebs solution cooled to ~15°C. Many ion channels are sensitive to temperature (Kito and Suzuki, 2007); this may have influenced the electrophysiological properties of smooth muscle cells. Preparations are also cut open and stretched during dissection and therefore physical damage may activate processes, which lead to uncoupling. I tested whether an influx of Ca^{2+} caused by damage during dissection might account for both uncoupling and hyperpolarisation of the cells' resting membrane potential. Cytosolic Ca^{2+} concentration can modulate gap junction conductance, via a calmodulin dependent protein kinase (Bloomfield and Völgyi, 2009). Smooth muscle cells also express several types of Ca^{2+} dependent K^{+} channels (Vogalis, 2000).

Damage from dissection may release various inflammatory mediators. Prostaglandins are synthesised in gastrointestinal tissues in response to damage by cycloxygenase COX 1 and COX 2 (Fornai et al., 2010, Porcher et al., 2002). Inhibition of these enzymes by indomethacin results in enhanced contractility in muscle strip preparations (Sanders, 1984). In the guinea pig ileum, indomethacin induced regular spontaneous contractions at a slow wave frequency in flat sheet preparations devoid of longitudinal muscle and myenteric plexus (Maggi et al., 1994b). To test if release of prostaglandins contributed to the loss of coupling, I inhibited prostaglandin synthesis before dissection commenced. Mast cells are involved in wound healing and defence against pathogens. There are two types of mast cells in the GI tract: mucosal mast cells and connective tissue mast cells (Bauer and Boeckxstaens, 2004). The majority of these cells are within the muscularis mucosa (Mota et al., 1956). Mast cells release granules containing inflammatory mediators such as histamine, heparin, cytokines, prostaglandins and leukotrienes upon activation. Accordingly, I have tested whether mast cell stabiliser affected dissection-induced loss of coupling.

In different cellular systems, a number of cellular changes can modulate gap junction conductance. Voltage and pH dependent gating have been demonstrated in biophysiocal studies on gap junction permeability, however their relevance to the present situation is uncertain (Goldberg et al., 2004). In other cellular systems, gap junction formation and function can be modulated by phosphorylation (Lampe and Lau, 2004). Elevated concentrations of cyclic adenosine monophosphate (cAMP) withn cardiac myocytes increase gap junctions conductance, while increased concentrations of cyclic guanosine 3′-5′-monophosphate (cGMP) decrease conductance (Burt and Spray, 1988).

This study aimed to determine the elements of dissection that account for gap junction uncoupling and to assess which mediators may activate the process.

4.2 Methods

The general experimental protocol is as described in chapters 2 and 3 with modifications as listed below. For each of the modifications, a separate series of experiments was conducted.

4.2.1 Experimental design

In previous experiments, preparations were dissected in Krebs solution chilled to ~15°C but recordings were made at ~35°C. To test if temperature per se affected recovery, responsive preparations were exposed to Krebs solution at 15°C for 5 and 30 minutes, the latter exceeding the duration of the entire initial dissection period. Intracellular recordings commenced once preparations were returned back to recording temperature (35°C) since neurotransmitter release, and therefore the amplitude of inhibitory junction potentials are affected by reduced temperature (Lang, 1979). To test the effects of cutting, two rows of gold pins were placed around the circumference of the tissue. The preparation is then allowed to recover for 120 minutes and control data was collected. Preparations were re-cut between the two rows of pins, creating a new edge around the entire circumference. Recordings after cutting were made from cells more than 1 mm from the boundaries to ensure that the recordings were not made from smooth muscle cells that had been directly cut. To test the effects of circumferential stretch, an array of hooks was placed along one longitudinal axis. Preparations were stretched to 115, 130 and 150% of it original resting length for 5 minutes, then returned back to resting length for recordings after the preparation had become fully responsive.

To test whether the loss of responses was due to Ca^{2+} influx from the Krebs solution during initial setup, preparations were dissected in solution containing low [Ca^{2+}], high [Mg^{2+}] (0.25mM and 10mM respectively) then transferred to normal Krebs solution for recording since neurotransmitter release requires the presence of

extracellular Ca^{2+}. Dissection in Ca^{2+} free Krebs solution with 1mM ethylenediaminetetraacetic acid (EDTA) was also trialled. The effects of prostaglandins, mast cells and cAMP during dissection were tested by preincubating the preparations in Krebs solution containing either: 3μM indomethacin (a non-selective inhibitor of cyclooxygenases COX 1 and COX 2); 10μM ketotifen (a mast cell stabiliser); or 10μM forskolin (which increases cAMP levels). Recordings were made in normal Krebs solution in each case. To test the effects of cGMP on gap junction permeability, control responsive cells were exposed to 100μM 8 bromo cGMP.

The effects of minimising net damage during dissection were also considered. Intracellular recordings from circular muscle cells were made from a group of preparations where the mucosa was kept intact. Cells were impaled via the serosa and longitudinal muscle layer. Responses to exogenous ATP and dye coupling could not be measured in these preparations due to poor visibility through the mucosa.

4.2.2 Drugs

Drugs included: EDTA stored at 10^{-1}M in aqueous solution used at final concentration of 1mM; indomethacin stored in DMSO at 10^{-2}M used at final concentration of 3μM; ketotifen stored in aqueous solution at 10^{-2}M used at final concentration 10μM; forskolin stored in DMSO at 10^{-2}M used at final concentration of 10μM and 8-bromo cGMP stored in aqueous solution at 10^{-1}M used at final concentration of 100μM. All drugs were purchased from Sigma Aldrich (Sydney, Australia).

4.2.3 Data analysis

Results are expressed as means± standard error of mean and "*n*" refers to the number of preparations. Two-tailed *t*-tests were used to compare paired or unpaired data samples. Linear regression analysis was used to compare the development of responses over time using Microsoft Excel (2004). One-way ANOVA was used to

compare the effects of varying degrees of stretch versus control, using PRISM software (Prism 4 for Macintosh, USA, 2003). For comparisons between mucosa removed versus mucosa intact, data previously presented in chapter 2 were used for mucosa removed samples.

4.3 Results

4.3.1 Temperature as a cause for initial suppression of responses

Reducing temperature for 5 minutes, after preparations had recovered full responses, did not cause a significant change in fast IJP amplitude, resting membrane potential (RMP), input resistance or dye coupling compared to the previously responsive conditions (n=6, Figure 4.1). This indicates that reduced temperature, per se, does not disrupt gap junction coupling. I also tested whether exposure to Krebs solution at 15°C for 30 minutes caused a loss of coupling. The longer period at reduced temperature also did not suppress responses. Fast IJP amplitude (-20.4±1.2 vs -17.5±6.0 mV), RMP (-55.2±0.7 vs -52.7±2.6), input resistance (4.9±1.7 vs 7.0±2.9) fast ,TTX-sensitive, ATP-evoked hyperpolarisation (-16.8±1.5 vs -16.4±2.6mV) and slow, TTX-insensitive, ATP-evoked hyperpolarisation (-8.5±1.8 vs -11.7±0.8mV) were not significantly different in cells impaled within 20 minutes of the temperature drop (n=3, Figure 4.2). Dye coupling reduced slightly (from 9.5±0.8 to 7.8±0.7 filled cells, P<0.05), however this does not compare with the near-abolition of coupling during the initial unresponsive period, after first setting up a preparation (n=3, Figure 4.2D). These results suggest that temperature, per se, is not substantively responsible for the loss of responses when preparations are initially set up.

4.3.2 Cutting a responsive preparation

Re-cutting the circumference of responsive preparations caused a significant reduction of responses, but not as extensive as the loss following initial dissection. In the first 20 minutes after cutting, the amplitude of fIJP was reduced to -9.6±1.5mV

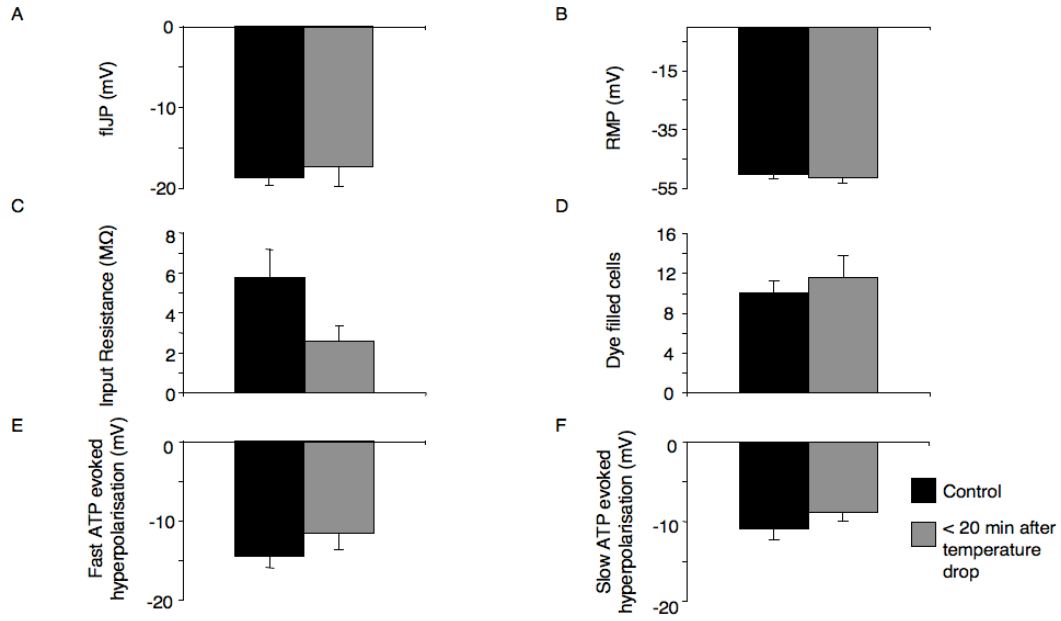


Figure 4.1 Exposure to Krebs solution at 15°C for 5 minutes did not induce a loss of responsiveness. There were no significant differences in: fIJP amplitude (A); RMP (B); input resistance (C) dye coupling (D); the amplitudes of the fast (E) and slow ATP evoked hyperpolarisations (F) (*n*=6).

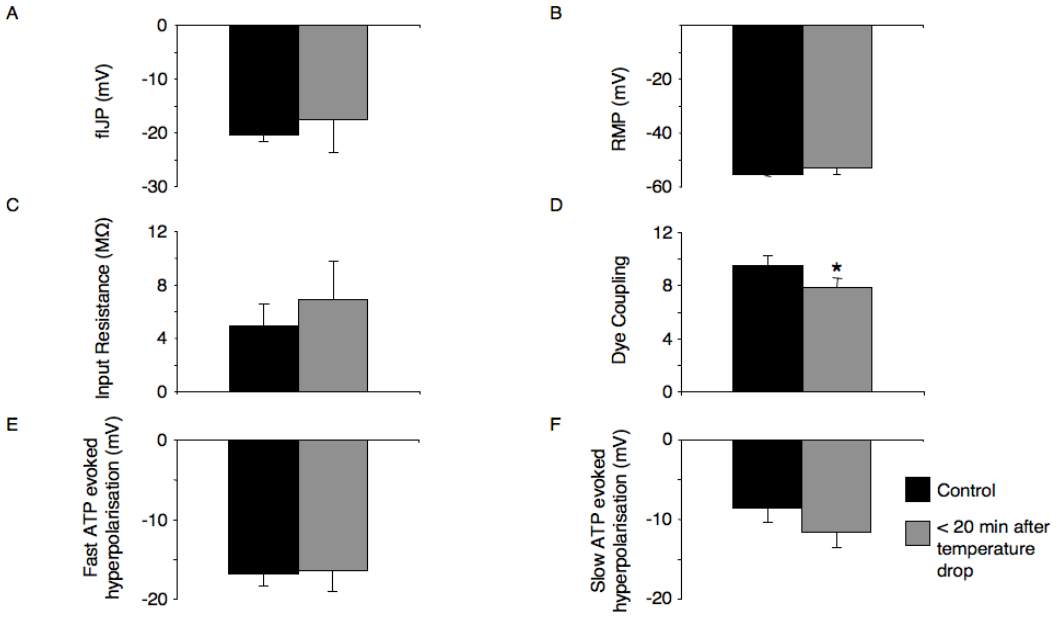


Figure 4.2 Exposure to Krebs solution at 15°C for 30 minutes did not induce a loss of responsiveness. Fast IJP amplitude (A), RMP (B), input resistance (C), amplitudes of fast and slow hyperpolarisation evoked by exogenous ATP (E and F) were not significantly different. D) Dye coupling was significantly reduced ($P<0.05$, *n*=3).

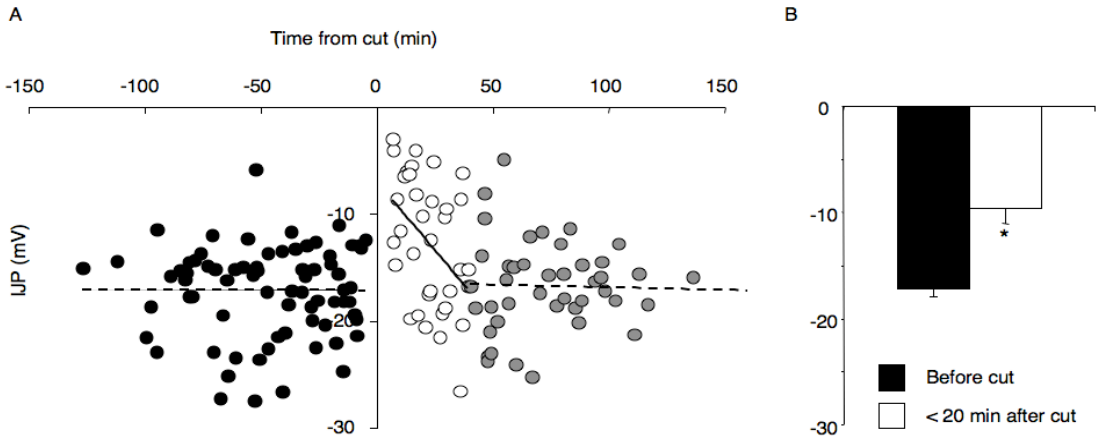


Figure 4.3 Re-cutting a responsive preparation reduced the amplitude of fIJPs. A) Filled circles indicate cells impaled before the cut had stable fIJP values (regression analysis not significant). Fast IJP amplitudes reduced after re-cutting and significantly increased within 40 minutes of re-cutting the preparation, open squares and dashed line (P<0.001, cells= 35, n=12). Fast IJPs did not significantly change after this (grey circles). B) Mean fIJP amplitude of cells impaled before re-cutting were significantly greater than cells impaled within 20 minutes of the cut (*, P<0.001,n=12)

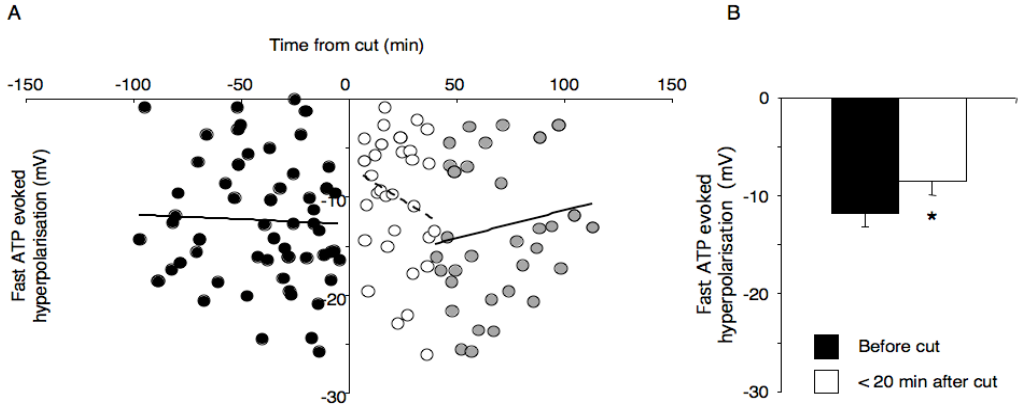


Figure 4.4 Re-cutting a responsive preparation reduced the amplitude of fast, ATP-evoked hyperpolarisations. A) Cells impaled before cutting showed no variation in the amplitude of hyperpolarisations (black circles). Amplitudes were initially reduced after re-cutting. They recovered over the next 40 minutes after cutting (white circles, not significant). Amplitudes did not significantly vary with time after the 40 minute recovery (grey circles, n=12). B) Cells impaled within 20 minutes of cutting had significantly smaller ATP evoked fast hyerpolarisations compared with cells impaled before (*P<0.05, n=12).

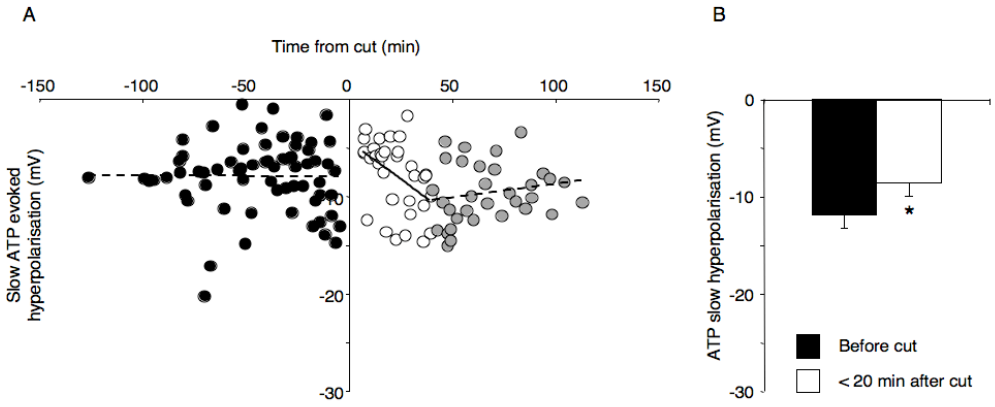


Figure 4.5 Re-cutting the edges of a responsive preparation reduced the amplitude of ATP-evoked TTX insensitive/ slow hyperpolarisations. A) Amplitude of cells impaled before cutting had stabilised and did not change significantly with time (black circles). After cutting, amplitudes were reduced and recovered significantly over 40 minutes (white circles, $P<0.01$, $n=12$). Amplitudes did not change after this. B) The amplitudes of ATP evoked slow hyperpolarisations were significantly greater before re-cutting compared to after ($P<0.01$, $n=12$).

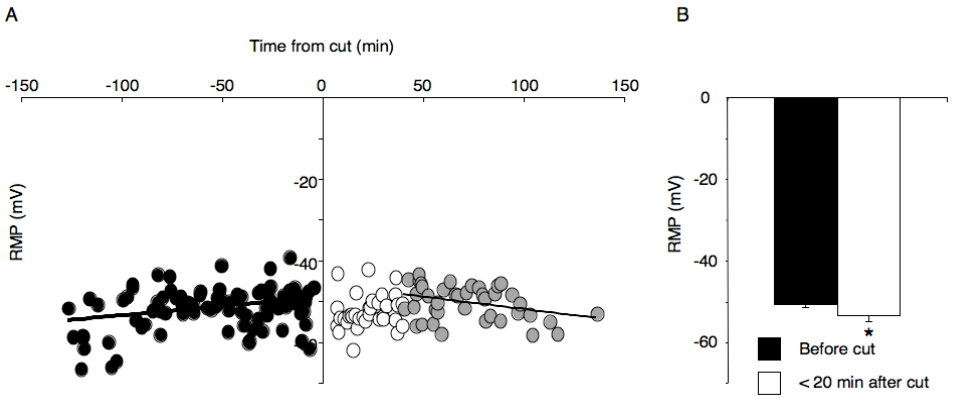


Figure 4.6 Re-cutting a responsive preparation hyperpolarised RMP of impaled cells. A) RMP in control cells slightly but significantly depolarised (black circles, $P<0.01$, $n=12$). RMP did not significantly change with time 40 minutes after cutting (white circles). RMP hyperpolarised significantly with time following the recovery period (grey circles, $P<0.01$, $n=12$). B) RMP of cells impaled within 20 minutes of re-cutting was significantly hyperpolarised compared to cells impaled before the cut ($P<0.05$, $n=12$).

from -17.2±1.5mV (P<0.0001, n=12, Fig 4.3B) but amplitude recovered over the next 40 minutes following the cut (P<0.01, n=12) and did not significantly change after this (Fig 4.3A). Fast-ATP evoked hyperpolarisations reduced from -12.3±1.3mV to -8.6±1.3mV within 20 minutes of re-cutting (P<0.05, Fig 4.4B) and recovered over the 40 minutes following the cut (Fig 4.4A). The amplitude of slow ATP evoked hyperpolarisations reduced to -8.6±1.3 mV from -11.8±1.4mV within 20 minutes after cutting (P<0.01, n=12, Fig 4.5B). Amplitudes recovered within 40 minutes after cutting (P<0.001, n=12, Fig 4.5A).

Mean RMP of cells impaled within 20 minutes of re-cutting was significantly hyperpolarised compared to cells impaled before the cut (-53.5±1.2 vs -50.6±0.7, P<0.05, n=12, Fig 4.6B). Input resistance did not significantly change following cutting (6.1±1.1MΩ pre cut, 5.9±0.9MΩ post cut, n=11, Fig 4.7B). Re-cutting reduced the mean number of dye filled cells to 7.4±1.0 profiles from 10.1±0.9 profiles following (P<0.05, n=12, Fig 4.8B) but this recovered over the 40 minutes following cutting (n=12, Fig 4.8A). Overall re-cutting the preparation caused a series of changes similar in direction to the original dissection, but of smaller amplitude and shorter duration. This suggests that damage to the original edges of the preparation may have contributed to the original loss of responses.

4.3.4 Stretching a responsive preparation

Stretching preparations to varying lengths had no effect on the responses of cells recorded in the first 20 minutes following the stretch: the amplitude of fIJPs; the amplitude of fast and slow ATP evoked hyperpolarisations; the RMP; the input resistance and the number of dye coupled profiles were not significantly different in control cells versus those impaled after stretching of up to 115, 130 and 150% of its resting length (Fig 4.9). Larger stretches were not possible as preparations began to tear with stretching up to 160% of it length.

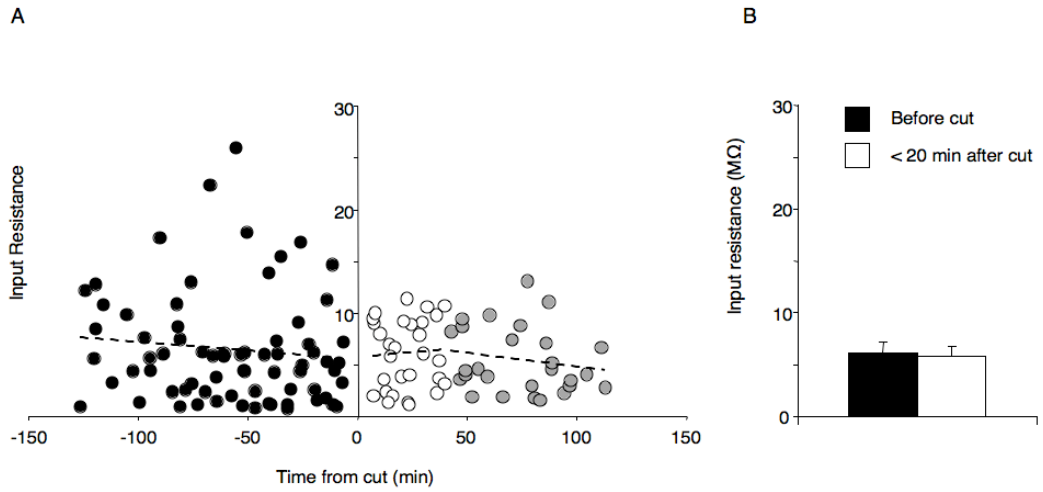


Figure 4.7 Re-cutting a responsive preparation did not affect input resistance. A) There were no significant changes in input resistance with time either before (black circles) immediately after (white circles) after 40 minutes following cutting (grey triangles, *n*=11). B) There was no significant difference in input resistance for cells impaled before or within 20 minutes after re-cutting (*n*=11).

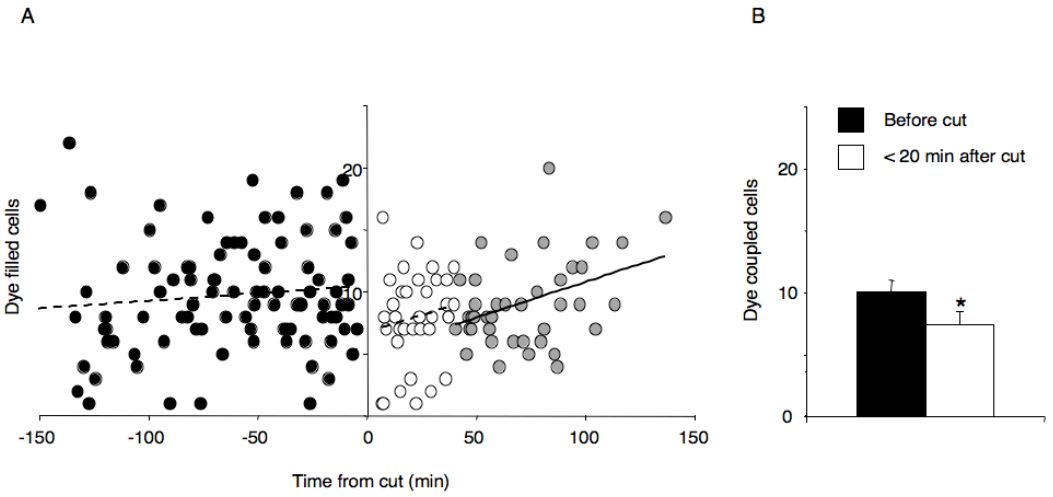


Figure 4.8 Re-cutting a responsive preparation reduced dye coupling with neighbouring cells. A) Number of dye filled cells reduced after re-cutting, then significantly increased with time following recovery (grey circles, *P*<0.005, *n*=11). B) Within 20 minutes of re-cutting, the number of dye filled cells significantly decreased compared to cells impaled before (*P*<0.05, *n*=12).

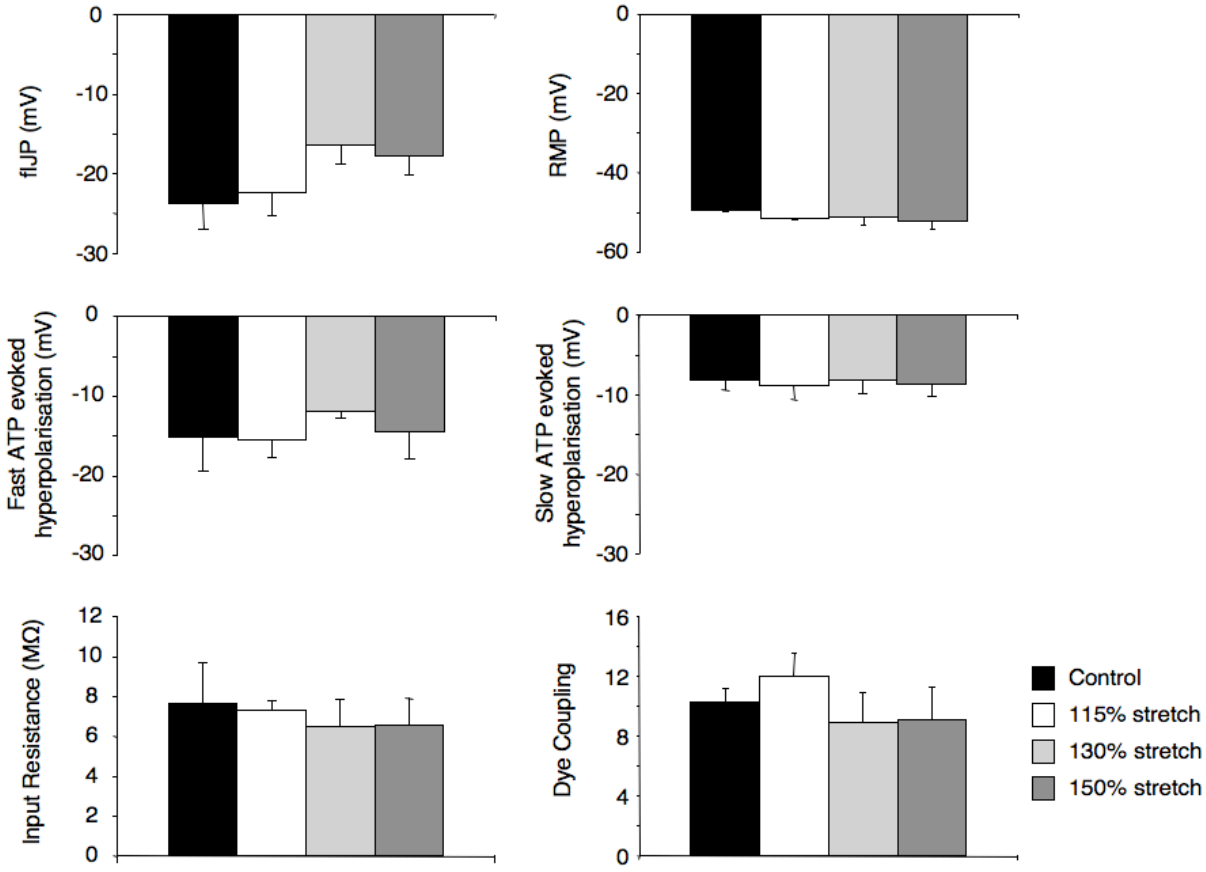


Figure 4.9 Responsive preparations were unaffected by high levels of stretch. Preparations were stretched 115, 130 and 150% of its original length. The amplitude of fIJPs (A), RMP (B), fast and slow ATP evoked hyperpolarisations (C and D), input resistance (E) and dye coupling (F) of cells within 20 minutes of stretching were not significantly different compared to controls measured before stretch (*n*=4).

4.3.5 Limiting influx of Ca^{2+} from Krebs solution during dissection.

There was a similar suppression of responses for preparations dissected in low Ca^{2+}, high Mg^{2+} Krebs solution as seen for preparations dissected in normal Krebs solution in chapter 2 (*n*=4, table 4.1). Mean fIJP amplitudes were significantly smaller in cells impaled within the first 30 minutes versus cells impaled after 120 minutes (-0.3±0.3mV vs -8.4±1.5mV, *n*=4, *P*<0.0001). RMP of cells impaled in the first 30 minutes was hyperpolarised compared to cells impaled after 120 minutes but this was not significant (-50.2±2.7mV vs -47.7±1.2mV, *n*=4). There were no significant differences in input resistance following dissection in low Ca^{2+}, high Mg^{2+} Krebs solution. Mean number of dye coupled cells was significantly greater in cells impaled after 120 minutes versus cells impaled within the first 30 minutes (4.5±0.5 versus 1.1±0.1, *P*<0.0001, *n*=4). Dissection in Ca^{2+} free Krebs solution with the Ca^{2+} chelator EDTA (1mM) was also tested. Although fast IJPs were ablated in the first 30 minutes, amplitudes and did not recover within or after 120 minutes (*n*=3) and so results were not considered further.

4.3.6 Prostaglandins as a mediator of uncoupling

The effects of prostaglandin release on the loss of responses was tested by preincubating for 40 minutes then dissecting preparations in indomethacin (3µM, table 4.1). Indomethacin had no effect on the loss of responses, since fIJP amplitudes within the first 30 minutes averaged 0±0.0mV and significantly increased to -14.1± 1.0mV (*P*<0.001, *n*=3) after 120 minutes. The amplitude of the fast ATP-evoked hyperpolarisation was negligible within the first 30 minutes and increased to -11.8±1.3 mV after 120 minutes (*P*<0.0001, *n*=3). The slow ATP-evoked hyperpolarisation was -0.2±0.4 mV in cells impaled in the first 30 minutes and increased to -7.4±0.5 mV after 120 minutes (*P*<0.001, *n*=3). The mean RMP for cells impaled in the first 30 minutes was -64.1±3.0mV. This was significantly hyperpolarised compared to cells impaled after 120 minutes (-49.9±0.5 mV, *P*<0.05, *n*=3). Mean input resistance did not show significant differences, however there was a trend that cells impaled within the first 30 minutes had greater mean input resistance compared to cells after 120 minutes (9.8±4.1 versus 5.3±0.5MΩ, *n*=3).

5.5±1.5 cells were dye filled in the first 30 minutes, compared to 10.0±0.8 cells filled after 120 minutes (P<0.05, n=3).

4.3.7 Stabilising mast cells to limit the loss of responses from dissection

Pre-incubating for 40 minutes and dissecting preparations in Krebs solutions with the mast cell stabiliser ketotifen (10μM) had no effect on the loss of responses following dissection (table 1). Fast IJPs had negligible amplitudes in cells impaled in the first 30 minutes and increased significantly 120 minutes after dissection (-18.8±2.4mV, n=3, P<0.05). Both fast and slow ATP evoked hyperpolarisations were negligible within the first 30 minutes following dissection. After 120 minutes fast ATP evoked responses were -10.8±1.0mV and slow ATP evoked responses were -6.5±0.9mV, significantly greater than responses within the first 30 minutes (P<0.05, n=3). The RMP of cells impaled 30 minutes from dissection were hyperpolarised compared to cells impaled after 120 minutes (-60.7±2.6mV to -48.4±1.2mV, P<0.05, n=3). The input resistance of cells impaled within the first 30 minutes of dissection was 14±3.0MΩ and decreased after 120 minutes to 10.0±3.1 (not significant, n=3). On average, 4.7±2.7 dye filled profiles were labelled from cells impaled within 30 minutes following dissection compared to 7.8±0.2 for cells impaled after 120 minutes (not significant, n=3).

4.3.8 Cyclic Adenosine monophosphate (cAMP) as modulator of gap junction coupling

In preliminary studies, the amplitudes of fIJPs in preparations pre-incubated in 10μM forskolin were negligible following dissection, while fIJP amplitude after 120 minutes averaged -5.9±0.8mV (n=3), much less than responsive cells from control preparations. Fast and slow ATP-evoked hyperpolarisations were negligible within 30 minutes of dissection and after 120 minutes increased to -5.8±3.0mV and -4.1±1.6mV, respectively. The RMP of cells averaged -50.8±2.2mV within 30 minutes of dissection and averaged -52.1±0.5mV after 120 minutes. Input resistance decreased from 11.3±2.3MΩ to 5.2±1.5MΩ. The mean number of dye coupled cells in the first 30 minutes was 4.3±1.9, compared to 8.7±2.8 after 120 mintes (n=3).

Table 4.1 The loss of responses of circular smooth muscle cells from preparations dissected in different solutions

	fIJP (mV)		RMP (mV)		Input resistance (MΩ)		Dye coupling		Fast ATP hyperpolarisation (mV)		Slow ATP hyperpolarisation (mV)	
	<30 min	**>120 min**	**<30 min**	**>120 min**	**<30 min**	**>120 min**	**<30 min**	**>120 min**	**<30 min**	**>120 min**	**<30 min**	**>120 min**
Control	-1.3±1.0	-15.8±0.9 †	-58.9±1.3	-47.7±0.6 †	14.4±2.2	8.4±0.7 *	2.0±0.3	4.2±0.3 †	0.0±0.0	-14.4±1.5	-6.0±0.6	-8.8±1.1
Low Ca^{2+}, High Mg^{2+}	-0.3±0.3	-8.4±1.5 ξ	-50.2±2.7	-47.7±1.2	48.9±19.8	10.3±4.1	1.1±0.1	4.5±0.5 ξ	-	-	-	-
Indomethacin (3µM)	0.0±0.0	-14.1±1.0 †	-64.1±3.0	-49.9±0.5 *	9.8±4.1	5.3±0.5	5.5±1.5	10.0±0.8 *	0.0±0.0	-11.8±1.3 ξ	-0.2±0.4	-7.4±0.5 †
Ketotifen (10µM)	0.0±0.0	-18.8±2.4 *	-60.7±2.6	-48.4±1.2	14.0±3.0	10.0±3.1	4.7±2.7	7.8±0.2	0.0±0.0	-10.8±1.0 *	0.0±0.0	-6.5±0.9 *
Foskolin (10µM)	0.0±0.0	-5.9±0.8	-50.8±2.2	-52.1±0.5	11.3±2.3	5.2±1.5	4.3±1.9	8.7±2.8	0.0±0.0	5.8±3.0	0.0±0.0	-4.1±1.6

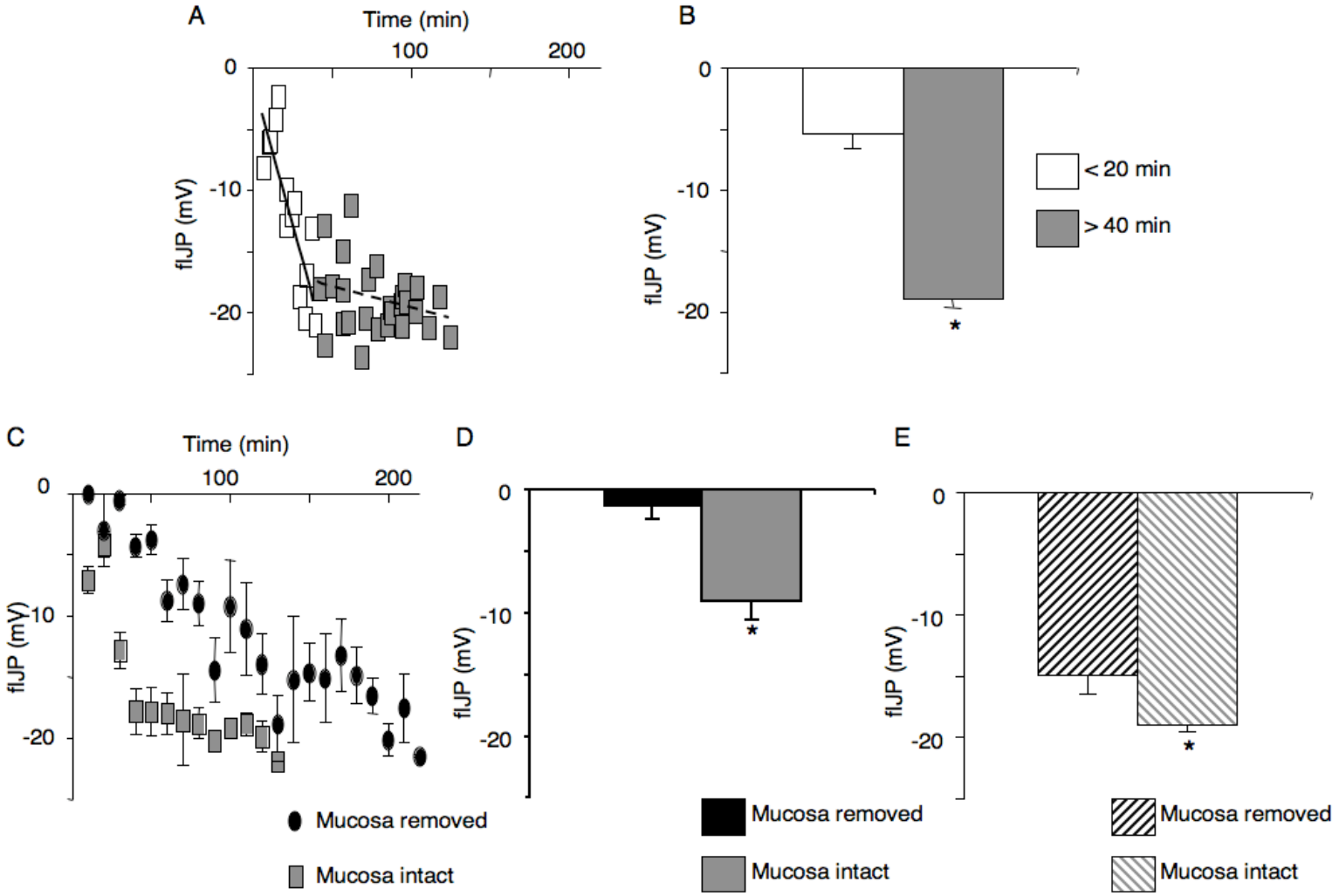
* $P<0.05$, § $P<0.01$, †$P<0.001$, ξ P,0.0001 comparison between values from cells impaled within 30 minutes of dissection versus after 120 minutes. Control n=12 (results from chapter 2) for ATP responses n=5 (results from chapter 3); low Ca^{2+}/ high Mg^{2+} n=4; indomethacin n=3; ketotifen n=3. Note: ATP-evoked responses were not tested during the low Ca^{2+} high Mg^{2+} experiments. A different microscope was used for experiments dissected in indomethacin, ketotifen and forskolin and accounts for the difference in number of dye coupled cells.

4.3.9 Cyclic guanosine 3′-5′-monophosphate (cGMP) as mediator of gap junction uncoupling

In a preliminary study (n=2) addition of membrane permeable 8-bromo cGMP (100μM) to responsive cells did not affect the amplitude of fIJPs in responsive cells (control: -13.6±1.4mV vs 8-bromo cGMP: -13.3±0.1mV). Similarly the amplitude of fast (-11.6±4.1mV) and slow (-8.1±0.7mV) ATP evoked hyperpolarisations were not affected by the addition of 8-bromo cGMP (fast: -11.6±0.2mV, slow: -7.3±0.8mV). The RMP of control cells was -47.2±0.9mV and -48.9±1.1mV in the presence of 8-bromo cGMP. The input resistance of cells reduced from 17.7±2.0MΩ to 12.5±1.9MΩ. Dye coupling in control cells was 7.2±0.2 labelled profiles compared to 5.9±1.5 profiles in the presence of 8-bromo cGMP (n=2).

4.3.10 The recovery of responses in partially dissected preparations with the mucosa intact.

In preparations in which the mucosa and submucosa were kept intact, the initial loss of responses was slightly reduced compared to fully dissected preparations where the mucosa and submucosa were removed prior to recording. Fast IJPs increased time dependently within 40 minutes following set up (P<0.005) and did not significantly increase with time after this (n=4, Fig 4.10A). Mean fIJP amplitude of cells impaled within 20 minutes was -5.4±1.2mV and this increased to -18.9±0.7mV in cells impaled after 40 minutes (P<0.005, n=4, Fig 4.10B). Fast IJPs of cells impaled within the first 30 minutes were significantly greater in these preparations (-8.8±1.5mV) compared to fully dissected preparations from chapter 2 (-1.3±1.0mV, P<0.005, n= 4 and 12 respectively). The RMP of cells impaled within the first 20 minutes was -58.8±2.0mV compared to after 40 minutes -55.2±2.0mV (not significant n=4, Fig 4.11B) For cells impaled within the first 30 minutes, there was no significant difference between preparations without the mucosa versus preparations with. Mean input resistance was not significantly different for cells impaled within 20 minutes of dissection (9.5±1.5MΩ) compared with cells impaled after 40 minutes (7.2±1.6MΩ). For cells impaled within the first 30 minutes, input resistance was significantly smaller in minimally dissected preparations (9.1±3.4MΩ) compared to cells from dissected preparations (14.4±2.2MΩ, P<0.05,


A
Time (min)
100
200
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-10
-20
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B
0
-10
-20
fIJP (mV)
< 20 min
> 40 min
*
C
Time (min)
100
200
0
-10
-20
fIJP (mV)
Mucosa removed
Mucosa intact
D
0
-10
-20
fIJP (mV)
*
Mucosa removed
Mucosa intact
E
0
-10
-20
fIJP (mV)
*
Mucosa removed
Mucosa intact

Figure 4.10 Fast IJPs were incompletely suppressed in partially dissected preparations. A) The amplitude of fast IJPs significantly increased within 40 minutes of dissection in mucosa-intact preparations (White squares, $P<0.005$, $n=4$). Amplitudes did not vary with time after this (grey squares). B) Mean amplitudes of fIJP for cells impaled 20 minutes after dissection where smaller than cells impaled after 40 minutes (* $P<0.005$, $n=4$). C) Mean fIJP amplitudes of cells binned in 10 minute intervals. D) Fast IJP amplitudes for cells impaled 30 minutes from dissection were greater in mucosa intact preparations (* $P<0.05$, $n=4$ and 12) E) Fast IJP amplitudes for cells impaled within 90-120 minutes were also greater in preparations with the mucosa intact (*$P<0.05$, $n=4$ and 12).

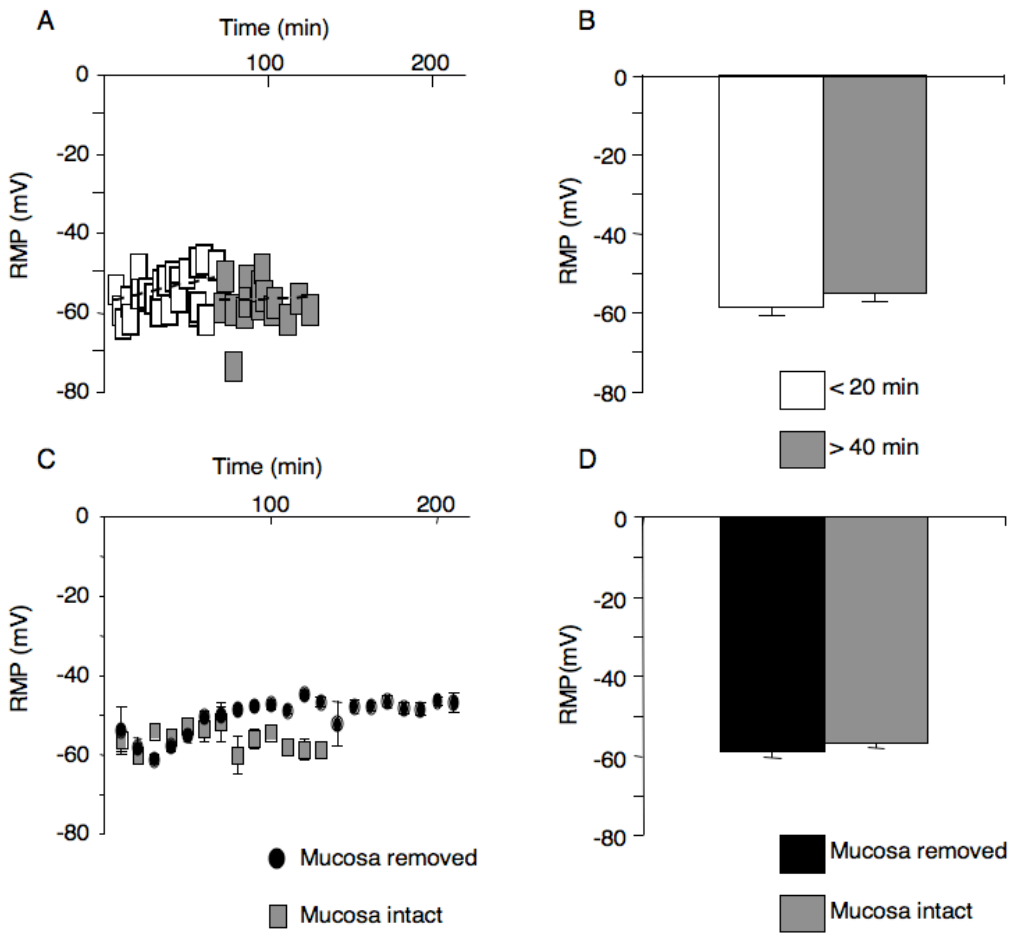

Figure 4.11 RMP of cells from mucosa intact preparations do not change following recovery from dissection. A) Cells did not significantly change within 40 minutes after dissection (white squares) or after this (grey squares). B) Mean RMP did not vary for cells that were impaled 20 minutes after dissection versus those impaled after 40 minutes (n=4). C) RMP averaged for cells in 10 minute bins. D) There was no significant difference between cells impaled 30 minutes from dissection for mucosa removed versus mucosa intact preparations.

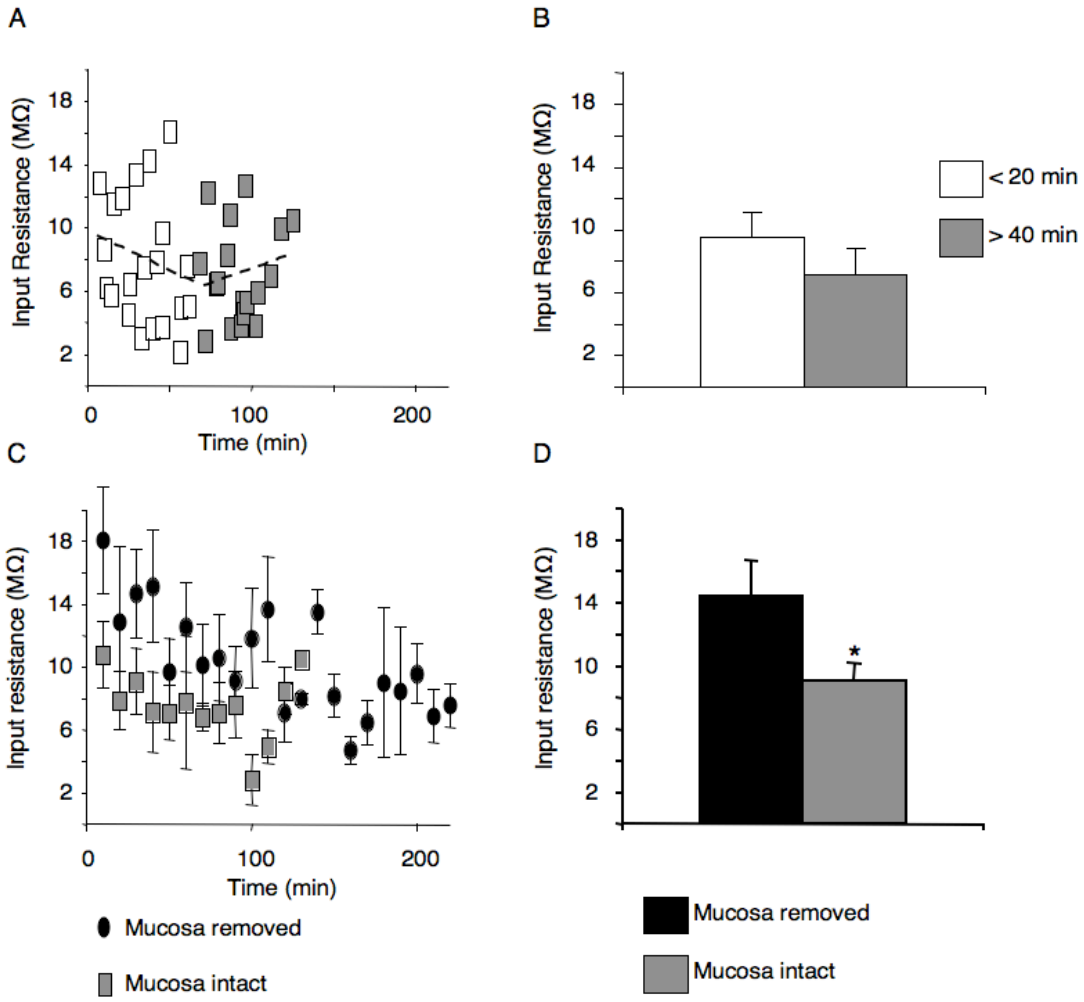

Figure 4.12 Input resistance did not change after dissection in mucosa intact preparations. A) For mucosa intact preparations, input resistance did not change over 40 minutes after dissection (white squares) or after this (grey squares) in partially (*n*=4). B) Input resistance was not significantly different for cells impaled within 20 minutes of dissection compared to cells impaled after 40 minutes (*n*=4). C) Mean input resistance for cells binned to 10 minute intervals; comparision of cells from mucosa on versus mucosa off preparations. D) Input resistance of cells impaled within 30 minutes of dissection was not significantly different between cells from mucosa intact versus mucosa removed preparations.

Fig 4.12D). Dye coupling could not be studied in these preparations due to poor visibility through the mucosa layer. Likewise, accurate placement of the micropipette for application of exogenous ATP was impossible, therefore these responses were not studied. Recordings of circular muscle cells in intact, tubular preparations were attempted, but was unsuccessful due to poor optics.

4.4 Discussion

In this study I have demonstrated that cutting a responsive preparation causes a second reduction of fIJP amplitudes that is associated with a slight reduction in gap junction coupling. However responses and coupling were attenuated less than occurs after initial dissection. None of the damage mediators which I tested were able to account for the initial loss of responses, however minimally dissecting specimens showed an incomplete suppression following setup. Reduced temperature and Ca^{2+} influx from Krebs solution did not account for the initial loss of response and uncoupling.

It is interesting to speculate on the mechanisms mechanical distortion/ damage activates that may affect gap junction coupling and tissue responsiveness. Little is known about the mechanisms by which gap junction permeability is regulated in inflammation and wound repair (Chanson et al., 2005), making it difficult to associate the physiological changes with mechanistic causes. Damage to tissue activates local release of inflammatory mediators such as prostaglandins. The mucosa also serves as a barrier protecting the body from potential pathogens that may enter via ingested content. While damage to the mucosa activates a range of pro-inflammatory processes that can affect gut physiology as displayed in diseases state such as inflammatory bowel disease (De Giorgio et al., 2004, Mawe et al., 2009) these processes occur over hours to days and require an intact blood supply. They do not occur at the same timescale as these studies. There is evidence that increase release of endogenous adenosine has anti-inflammatory affects, although the mechanism of actions is unknown(Colgan and Eltzschig, 2012, Cronstein, 1994). In the gastrointestinal tract, inflammation and hypoxia trigger the release of adenosine

(Colgan and Eltzschig, 2012), and the role of different adenosine receptors has been investigated in inflammatory bowel disease (Ren et al., 2011). A role for this metabolite in our system may also be worth considering.

4.4.1 Involvement of other factors

There are multiple sources of damage from setting up preparations for recording: the specimen is cut open along the mesenteric border and along the circumfrential axis to reduce it to an appropriate size; the mucosa and submucosa are pulled off by sharp dissection; the preparation is stretched to pin it to the base of the recording chamber. Cutting responsive preparations evoked a small loss of responses, but not to the same extent as the initial setup. While keeping the mucosa intact limited recovery time, there was still a suppression of responses following dissection. It may be that these two factors applied simultaneously are enough to result in the total loss of responses seen after dissection. However there is still a possibility that other mechanisms may be having influence. Surgical damage to tissue has been shown to immediately reduce local pH (Woo et al., 2004). Activation of clotting mechanisms by incision may lead to ischaemia and therefore decrease tissue pH. An alternative contribution to decreased pH may be from neutrophils and monocytes which enter the damaged regions and consume oxygen and release hydrogen ions (Woo et al., 2004). Reduced intracellular pH by acidifying Krebs solution, has been shown to decrease gap junction coupling between ICC-MY (Belzer et al., 2002), between ICC-DMP, and ICC-DMP to smooth muscle cells in the guinea pig ileum (Kobilo et al., 2003). My preparations were dissected in Krebs solution, a salt solution designed to mimic the extracellular conditions *in vivo*. The Krebs solution was equilibrated with medical carbenox which contains 5% CO_2 to buffer pH. Intracellular pH in smooth muscle cells is sensitive to changes in the CO_2 concentration (Aickin, 1984) and the initial exposure to Krebs solution may have an effect on the intracellular pH. It would be interesting to test the recovery of preparations exposed to different salt solutions, such as HEPES or modified Krebs solution with higher pH.

4.4.2 Limiting damage

While I was able to measure fIJPs in minimally dissected preparations with the mucosa intact, I was not able to further these investigations by measuring fIJPs in

intact tubular preparations. Intracellular recording is a desirable technique to use since dye coupling, a simple measure for gap junction coupling, can be easily applied. Impaling smooth muscle cells however is difficult in this preparation, therefore other methods to measure neuromuscular transmission may be considered in future studies. Recovery of contractility is one possibility. In chapter 2 I demonstrated that contractions recover following dissection and this recovery was faster in preparations where the mucosa was kept intact. Measuring contractions in intact tubular preparations is not difficult and future studies should consider the recovery of contraction following setup of this preparation.

4.4.3 Potential mediators of gap junction uncoupling

Indomethacin and ketotifen can affect the activity of intestinal smooth muscles (Abu-Dalu et al., 1996, Maggi et al., 1994b) but inhibiting prostaglandin synthesis and stabilising mast cells during dissection did not limit the loss of responses in these preparations. I postulated the involvement of mast cells due to their role in wound repair and their concentration at the circular muscle/ mucosa border (Mota et al., 1956). However, there are two types of mast cells: mucosal and connective tissue mast cells. There is some suggestion that ketotifen only stabilises connective tissue mast cells (de Jonge et al., 2004). Therefore a more general mast cell stabiliser, such as doxantrozole, may have been more effective in our experiments. There are multiple cell types at the sub-epithelial border with a role in inflammation and these cells may also influence responses following dissection. Macrophages are one such cell type. They secrete a range of substances, from cytokines, NO, prostaglandins, reactive oxygen intermediates and defensins (Bauer and Boeckxstaens, 2004). It would be interesting to limit the effects of these cells during dissection. Also, in this study the effects of indomethacin and ketotifen were trialled individually. A mixture of inhibitors applied simultaneously to block multiple damage mediators may be required for maximal effect.

Reagents produced by oxidative stress could be another potential mediator for uncoupling. For example exposure to the pesticide lindane results in oxidative stress

of myometrial smooth muscle cells which leads to uncoupling and decreased spontaneous contractility (Criswell and Loch-Caruso, 1999, Caruso et al., 2005). However whether this is relevant to this damaged induced supression is uncertain.

4.4.4 Phosphorylation and gap junction intercellular coupling

Gap junction conductance can be modulated by phosphorylation, therefore activation of secondary pathways may lead to loss of responses following dissection. Gap junctions are made up of connexins, these subunits are comprised of 4 domains within the membrane: 2 extracellular loops; an intracellular loop; and intracellular C and N termini. The C terminus is the main region for phosphorylation (Lampe and Lau, 2004). Within the smooth muscle layers of the gut, connexin 43 has been identified as the most abundant connexin, while connexin 40 and 45 are also present (Seki and Komuro, 2001, Wang and Daniel, 2001). Multiple protein kinases mediate cell coupling. It has been well documented that Cx 43 gap junction assembly and permeability is enhanced by increased cAMP levels, in other cellular systems (Spray and Bennett, 1985, Bloomfield and Völgyi, 2009). In my study, preincubation in forskolin had no effect on the loss of responses, nor on the reduced number of dye coupled cells, however the recovery of fIJPs was impaired and did not reach normal amplitude even after 120 minutes. Cyclic AMP is a second messenger and can activate cAMP-dependent protein kinases (PKA), therefore, it is not unexpected for elevated cAMP to have had multiple effects on our preparation. The increased levels of cAMP may have inhibited neurotransmitter release thereby limiting the amplitudes of fIJPs (King, 1994). The RMP of these cells was slightly hyperpolarised and this may be explained by elevated cAMP. Cyclic AMP-dependent processes control transient release of calcium from IP_3 operated stores, this can lead to the opening of Ca^{2+} activated K^+ channels, thereby hyperpolarising the cell (Hagen et al., 2006). This may have contributed to the impaired fIJP. Cyclic AMP dependent processes also inhibit voltage sensitive Ca^{2+} channels, and also could have contributed to the cell hyperpolarisation (Makhlouf and Murthy, 1997).

Gap junction coupling can be limited by modulating conductance of a connexin channels. However, gap junctions have a half life of a few hours (Laird, 2006). Uncoupling mechanisms may also affect the turnover of gap junctions. I am yet to

determine whether uncoupling from dissection specifically affects gap junction conductance or gap junction cycling. To approach this problem I would need to apply a combination of Western Blot analysis to measure levels of connexin expression with immunohistochemistry and freeze fracture analysis to determine whether connexin expression is on the cell membrane and if gap junctions are formed (Mori et al., 2005). As indicated in chapter 3, electrophysiological recordings can also give a reflection of dynamic changes in gap junction coupling. However, these are necessarily confounded by changes in smooth muscle membrane resistance and would not be a reliable measure of gap junction permeability, per se

4.4.5 Clinical applications

It is interesting to speculate whether the mechanisms which cause the loss of responses following dissection also result in the occurrence of post operative ileus. Post operative ileus is a condition which arises following abdominal surgery that leads to impaired gastrointestinal motility; not just a loss of peristalsis but hypomotility of the entire tract (de Jonge et al., 2004). This condition occurs after many abdominal surgical procedures and typically lasts 2-4 days. It can progress to prolonged or paralytic ileus when duration extends more than 6 days (Boeckxstaens and De Jonge, 2009). Worth noting, minimally invasive surgery with laproscopic techniques results in shorter duration of post operative ileus (Bauer and Boeckxstaens, 2004). Multiple mechanisms contribute to post operative ileus. Extrinsic neural inputs are activated within the first few hours following surgery, inflammatory mechanisms involving mast cells and macrophages are activated after surgery and last for days and the inflammatory substances released will affect the activity of local intrinsic neurons (Bauer and Boeckxstaens, 2004). In my experiments I did not test the effects of surgically removing the specimen from the animal. This would be difficult to do and different experimental measures would need to be considered to achieve this. Studying intact tubular preparations would be helpful in answering whether responses are lost once the specimen is removed from the animal. However, only in vivo studies would confirm whether the activity is present initially and then lost. An electrode array can be applied to the bowel in vivo to measure peripheral pacemaker activity and the propagation of events can be

monitored (Lammers et al., 2005). Following this the specimen could potentially then be removed from the animal and the change in responses may be monitored. It is also worth considering that in a study investigating the role of mast cells in post operative ileus, animals were administered the stabilisers by IP injection or by oral gavage for 5 consecutive days before the surgery. These stabilisers were effective in reducing hypomotility after surgery (de Jonge et al., 2004).

4.5 Conclusion

Exposure to reduced temperatures and influx of Ca^{2+} from Krebs solution did not contribute significantly. There was clearly a role of mechanical damage in causing the unresponsive period, but the loss and subsequent regeneration of responses remained when prostaglandin synthesis was inhibited and mast cells were stabilised. While tissue damage has therefore been implicated as a cause of uncoupling, the intracellular mechanisms activated by mechanical damage to intestinal muscle remain to be identified.