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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3. EVIDENCE FOR THE INVOLVEMENT OF GAP JUNCTION COUPLING IN NEUROMUSCULAR TRANSMISSION

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3.1 Introduction

In many regions of gut, smooth muscle cells are coupled to one another to form a functional syncytium. This coupling is mediated largely by gap junctions. Gap junctions are formed by pairs of hemichannels or connexons, which allow passage of ions and small molecules up to a molecular weight of 1000Da. Each connexon is made up of six subunits (connexins), which determine the properties of the channel. Gap junctions underlie electrical coupling and metabolic coupling of cells in the smooth muscle apparatus and are the structures by which cells are dye-coupled (Weber et al., 2004). Their role in neuromuscular transmission and smooth muscle contractility has been debated (Sibaev et al., 2006, Daniel et al., 2007, Daniel, 2004). Several studies have identified specific connexins in the smooth muscle layers of the gut, with Cx43 being most abundant; Cx40 and Cx45 have also been shown to be present (Wang and Daniel, 2001). The connexins may be differentially located, with Cx45 being preferentially associated with junctions between interstitial cells of Cajal (ICC) (Seki and Komuro, 2001).

In chapter 2 I demonstrated that inhibitory and excitatory junction potentials recorded from circular muscle cells are diminished following dissection, however excitatory post synaptic potentials recorded in neurons persisted. The loss of junction potentials was associated with a hyperpolarised resting membrane potential (RMP), reduced dye coupling and increased input resistance. This led to the hypothesis that loss of junction potentials was due to a post junctional change, most likely gap junction uncoupling,

caused by the process of setting up the preparation. This study aimed to assess the effects of gap junction blockers on tissue excitability and dye coupling in the guinea pig ileum and colon. The responses of circular smooth muscle cells to exogenous ATP, a potential mediator for fast inhibitory junction potentials (fIJPs), was measured during recovery from dissection.

3.2 Materials and Methods

Materials and methods are as described in the previous chapter with the following additions.

3.2.1 Electrophysiological responses to exogenous ATP

Circular smooth muscle cells were recorded from as previously described in chapter 2. Adenosine triphosphate (ATP, 10mM, in Krebs solution) was locally pressure ejected with nitrogen pulses (140kPa, 20-50ms) applied to the back of a 1mm borosilicate pipette, broken to have a ~10µm diameter tip. For the sake of consistency, ATP was used throughout these experiments, rather than trying related molecules (eg: ß-NAD) that may mediate fast inhibitory junction potentials or P2Y1 receptor agonists. The pipette tip was placed 100µm circumferential to recording site: any closer and I risked the puff from the pressure ejection dislodging the electrode from the recorded cells; any further away and smooth muscle cells sometimes failed to generate reliable responses. Inert blue food dye (Famous Queen, Australia, 0.01%) was used to visualise the trajectory of the ejected fluid to ensure it passed over the area of the recorded cell.

Following recovery from dissection (>120 minutes) the effects of a series of gap junction blockers on neuromuscular transmission were trialled. Drugs used included: carbenoxolone; stored at 10^{-2}M in aqueous solution used at final concentration of 100µM, 18ß glycyrrhetinic acid; stored at 10^{-1}M in ethanol, used at final concentration of 10µM and 2-Aminoethoxydiphenyl Borate (2-APB); stored at 10^{-1}M in DMSO used at final concentration 50µM. All drugs were purchased from Sigma Aldrich (Sydney, Australia).

3.2.2 Visualising carboxyfluorescein labelling in sections

In a separate series of experiments, circular smooth muscle cells were dye filled at different times following dissection. Preparations were sectioned so that the spread of dye to cells above and below the plane of the filled cell could be visualised. Multiple specimens were prepared from ileum of 4 animals. One to two cells were filled per preparation using the same dye injection method as chapter 2. Preparations were fixed overnight using a modified Zamboni's fixative (0.2% saturated picric acid and 2% paraformaldehyde in phosphate buffer). They were then washed with phosphate buffered saline (PBS) 3 times, cleared with dimethyl sulfoxide (DMSO) 3 times and then rewashed 3 times in PBS- each process for 10 minutes. In preparation for cryostat sectioning, specimens were placed into 30% sucrose (in PBS) overnight and stored at 4°C. Preparations were then embedded in OCT compound, oriented for either sectioning along the longitudinal or circumferential axis, and were frozen in this solution. Blocks were cut on a cryostat and serial 12µm sections were taken. Sections were mounted onto Vectabond-treated slides (Vector Laboratories, Burlingame, CA), and were viewed on an Olympus IX71 epifluorescence microscope (Japan) equipped with highly discriminating filters (Chroma Technology Co., Battledore, VT). Images were captured using a Roper scientific (Coolsnap) camera using AnalySIS Imager 5.0 (Olympus-SIS, Münster, Germany).

3.2.3 Immunohistochemistry

In a separate series of experiments, specimens of guinea pig ileum from 3 animals were fixed and cleared in the method described in section 3.2.2. These preparations had not been dye filled. Following washes in PBS, preparations were incubated in blocking solution for 1 hour (20mg/mL bovine serum albumin, 5% normal donkey serum, 95% PBS Triton-X) at room temperature. This was to reduce background labeling. Specimens were incubated in primary antibodies overnight at room temperature, rinsed with PBS, then incubated in secondary antibodies for 3 hours. The primary antibody used was rabbit anti platelet derived growth factor receptor alpha, from Abcam used at 1:200 (PDGFRα, cat. no. ab61219, Waterloo, NSW, Australia). This antibody was used

to label 'fibroblast like cells' which are thought to be an intermediary cell mediating purinergic transmission between inhibitory motorneurons and smooth muscle cells (Kurahashi et al., 2012, Kurahashi et al., 2011). The secondary antibody, used at 1:400, was CY3- donkey anti-rabbit (Jackson, cat. no. 74548, West Grove, USA).

3.2.4 Measuring the force of contractions

In another series of experiments on preparations of guinea pig colon, basal tone of preparations, the amplitude of spontaneous contractions and the area under the curve from 20s epochs were measured over the course of recordings (as described in chapter 2). Following 120 minutes from dissection, control data was collected and the responses of preparations were compared with control and following the addition of carbenoxolone (100μM) and sequential addition of TTX (0.6μM).

3.2.5 Data Analysis

Results are expressed as means ± standard error of mean and '*n'* refers to the number of preparations. Student's two-tailed paired *t* tests were used to compare control versus test samples. Linear regression analysis was used assess the development of ATP evoked hyperpolarisations with time. All data was analysed using Microsoft Excel (2004). For force measurements, the values for each 20s epoch were averaged over the course of the given experimental condition. 2-way ANOVA was used to compare the difference between means (Prism 4 for Macintosh, USA, 2003).

3.3 Results

3.3.1 Effects of gap junction blockers on the excitability of circular muscle cells.

The effects of gap junction blockers, carbenoxolne, 18ß glycyrrhetinic acid and 2-aminoethoxydiphenyl borate (2-APB) were tested on circular muscle cells after full responses had developed, to determine if pharmacologically reducing gap junction coupling altered neuromuscular transmission. In the presence of 100μM carbenoxolone, fIJPs were reduced significantly from a control value of -14.6 ± 1.2mV to -7.8± 1.3mV,

P<0.005, *n*=6. Carbenoxolone (100µM) induced a small but significant hyperpolarisation of circular muscle cells from -47.0±0.5mV in controls to -49.3±0.5mV (*P*<0.05, *n*=6) and apparent input resistance increased from 8.9±1.7MΩ to 30.8±4.0MΩ (*P*<0.005, *n*=6). Correspondingly, dye coupling in equilibrated circular muscle cells decreased from 4.4±0.5 cells to 1.2±0.1 cells (*P*<0.005, *n*=6, see table 3.1).

Similar results were obtained with 10µM 18ß glycyrrhetinic acid (18ßGA). Mean fIJP amplitude was reduced from -14.0±1.8mV to -6.2±1.1mV (*P*<0.05, *n*=6). Cells were not hyperpolarised by 10µM 18ß glycyrrhetinic acid (-50.6±1.4mV compared to -49.5±0.6mV for control, *n*=6, not significant) but input resistance increased from 8.5±0.6MΩ in control to 111.5±40.4MΩ (*P*<0.05, *n*=6) in the gap junction blocker. Correspondingly, dye coupling was reduced from 5.7±0.6 cells to 1.2±0.07 cells (*P*<0.001, *n*=6, see table 3.1).

Table 3.1: The effects of gap junction blockers on ileal circular smooth muscle cells impaled 120 minutes after dissection.

	Control	Carbenoxolone (100µM)	Control	18ß Glycyrrhetinic Acid (10µM)	Control	2-APB (50µM)
fIJP amplitude, mV	-14.6±1.2	-7.8±1.4§	-14.0±1.8	-6.2±1.1 *	-16.1±0.8	-0.6±0.5†
RMP, mV	-47.0±0.5	-49.3±0.5*	-49.5±0.5	-50.5±1.4	-50.4±0.5	-54.1±1.4
Input Resistance, MΩ	8.9±1.7	30.8±4.0§	8.6±0.6	111.5±40.4 *	7.5±1.0	140.3±52.0
Dye coupling	4.4±0.5	1.2±0.1§	5.7±0.6	1.2±0.1 †	9.8±0.2	1.9±0.4‡

Values are means ±SEM; n=6 (carbenoxolone and 18ß-glycyrrhetinic acid) and 4 (2-APB). *P<0.05; †P< 0.001; §P<0.005; ‡P< 0.0001 vs. respective control.

2-APB has been shown to block gap junctions (Bai et al., 2006) including those comprised of connexin 43 (Yang et al., 2011). 2-APB significantly reduced fIJP amplitude (-16.1±0.8mV to -0.6±0.5mV $P<0.001$, $n=4$). RMP of cells displayed a non-significant trend to hyperpolarisation in the presence of 2-APB, (-50.4±0.5mV to -54.1±1.4mV, $n=4$, $P=0.08$). Input resistance increased in the presence of 2-APB, (7.5±1.0MΩ to 140.3±52.0MΩ, $n=4$, $P=0.08$). It should be noted that these results may have been distorted by one outlying value and the low number of specimens ($n=4$). 2-APB significantly decreased dye coupling from 9.7±0.2 cells to 1.9±0.4 ($P<0.001$, $n=4$, see table 3.1).

The effects of gap junction blocker carbenoxolone (100µM) on colonic smooth muscle cells were measured. The amplitude of the fIJP was reduced from -27.6±2.5mV to -6.3±1.mV by carbenoxolone ($P<0.005$, $n=5$, Fig 3.1A). Carbenoxolone caused the RMP of cells to significantly hyperpolarise from -43.3±0.7mV to -47.6±1.4mV ($P<0.05$, $n=5$, Fig 3.1B). Apparent input resistance was increased from 8.6±0.4MΩ to 47.1±9.5MΩ by the presence of 100µM carbenoxolone ($P<0.05$, $n=5$, Fig 3.1C). Dye filled profiles averaged 6.7±0.6 cells in control conditions and decreased to 1.3±0.08 in carbenoxolone ($P<0.001$, $n=5$, Fig 3.1D).

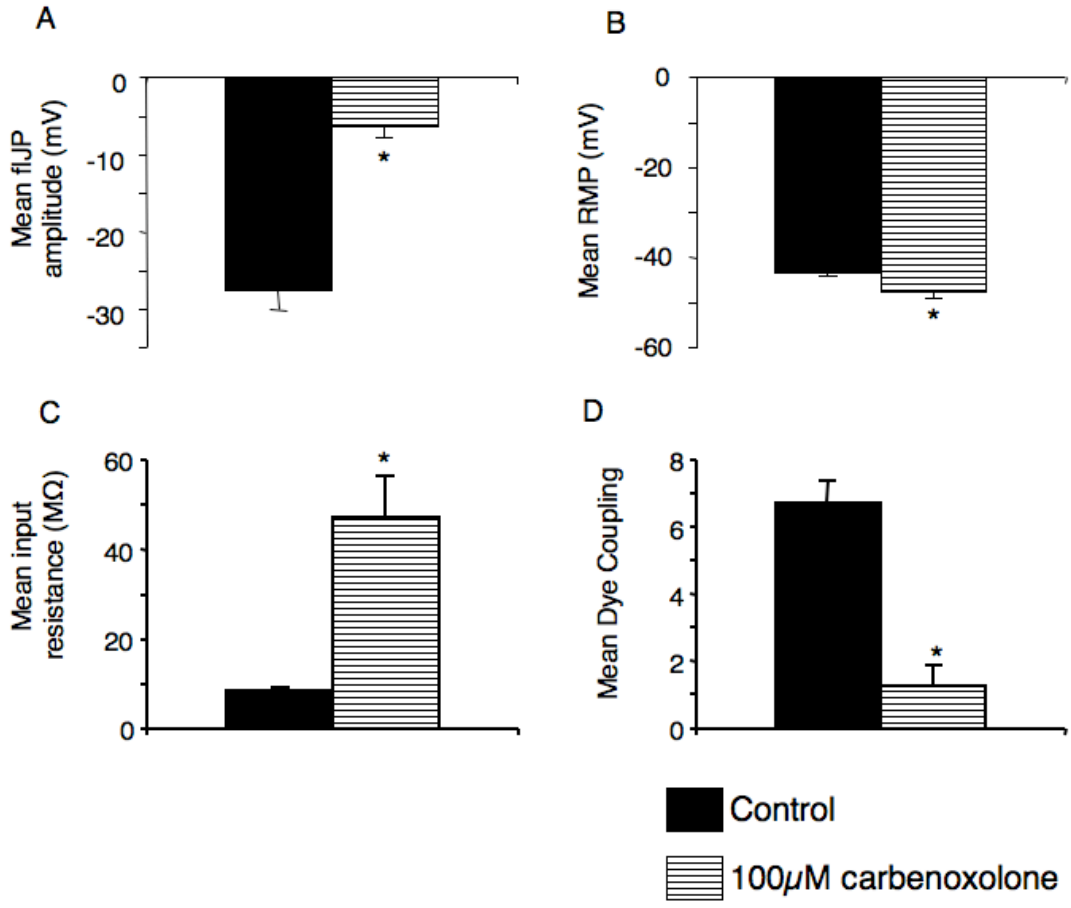


Figure 3.1: The effects of 100µM carbenoxolone on colonic smooth muscle cells. Addition of 100µM carbenoxolone 120 minutes after dissection A) reduced the amplitude of fIJP (*P<0.005, n=5) *B)* hyperpolarised RMP (* P<0.05, n=5) *C)* increased input resistance (*P<0.05, n=5) and D) decreased dye coupling (* P<0.001, n=5).

3.3.2 The effects of carbenoxlone on slow inhibitory junction potentials (sIJP) and excitatory junction potentials (EJP)

Addition of 100μM carbenoxolone significantly reduced mean sIJP amplitude from -2.5±0.1mV to -0.13±0.3mV in ileal smooth muscle cells (P<0.005, n=4, Fig 3.2A) when stimulated with 3 pulses at 50Hz. In colonic smooth muscle cells, addition of 100μM carbenoxolone significantly reduced EJP amplitude from 8.9±3mV to 2.5±0.9mV (P<0.05, n=5, Fig 3.2B).

3.3.3 Effects of exogenous ATP

The fIJP in circular muscle cells of the guinea pig ileum is mediated by ATP (Crist et al., 1992) or a related molecule such as nicotinamide adenine dinucleotide (NADH (Mutafova-Yambolieva et al., 2007)), largely via P2Y1 receptors (Wang et al., 2007). I tested whether changes in fIJP amplitude were associated with post-junctional changes by examining the effects of exogenous ATP applied by pressure microejection onto circular muscle cells. In circular muscle cells in which fIJP amplitude had stabilised, pressure-ejected ATP typically evoked a biphasic hyperpolarisation, in which the first, larger phase resembled the fIJP in time-course. This was followed by a later, slower hyperpolarisation (Fig 3.3A). In circular muscle cells impaled after the first 120 minutes, the amplitude of the fast component was -14.4±1.5mV and the slow component was -8.8±1.1mV (n=5, Fig 3.3C). The first phase was blocked by 0.6μM tetrodotoxin, but the second phase persisted, with slightly reduced amplitude (-6.0±0.6mV, Fig 3.5A, n=6). This suggests that the first component was due to activation of axons of inhibitory motor neurons which then evoked a true fIJP where as the second, TTX-resistant phase, was due to direct actions of ATP on the muscular apparatus.

Both fast and slow components of ATP responses were significantly smaller in cells during the first 30 minutes of recording (0.0±0mV, -0.6mV±0.6mV, respectively, P<0.05, n=5, Fig 3.3B). Carbenoxolone (100μM) added after preparations had developed full responses significantly reduced the amplitude of both ATP hyperpolarisations (fast component reduced from -13.8±2.4 to -3.7±1.4mV; slow

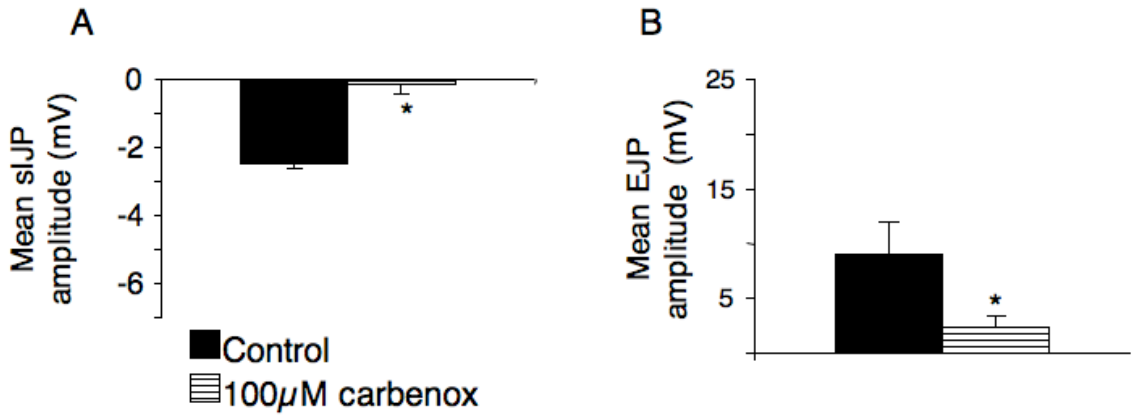


Figure 3.2: Addition of 100μM carbenoxlone significantly reduced the amplitude of junction potentials. In responsive ileal circular muscle cells (cells impaled after 120 minutes), A) sIJPs (* $P<0.005$, $n=5$) and B) EJPs (* $P<0.05$, $n=5$) were reduced following addition of carbenoxolone. Note: the amplitudes of sIJPs and EJPs for circular smooth muscle cells impaled within 30 minutes of dissections were 0.0±0.0mV and 1.8±1.1mV, respectively (Section 2.3.4 and 2.3.5).

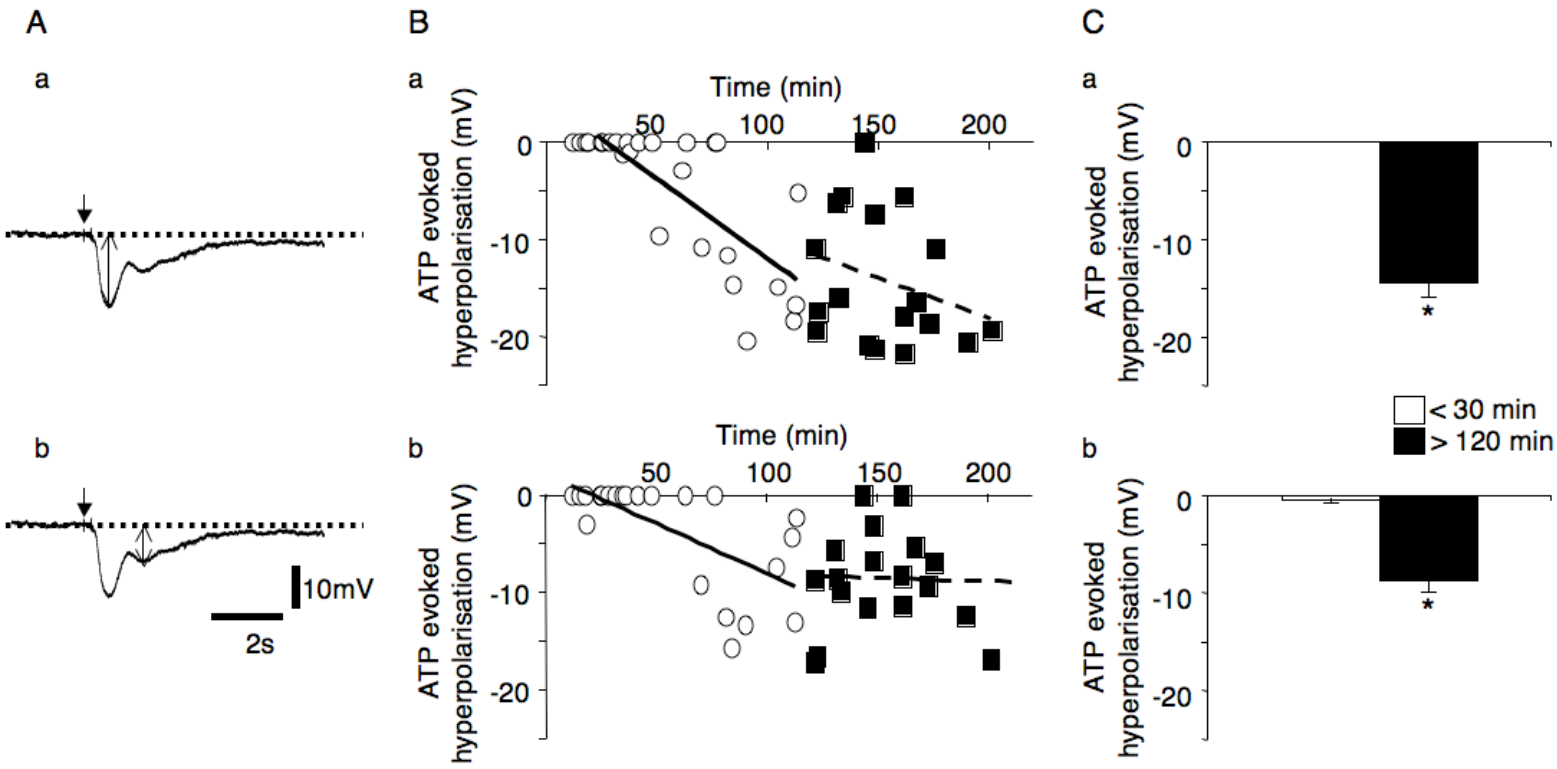


Figure 3.3: Responses to exogenous ATP were biphasic in ileal smooth muscle cells. A. Application of 10mM ATP (closed arrow) generated a biphasic response with a fast IJP like component (Aa, open arrow) followed by a slow hyperpolarisation (Ab, open arrow). The fast (Ba) and slow (Bb) components had time dependent increases in the first 120 minutes ($P<0.001$, $n=5$). The fast (Ca) and the slow (Cb) components were significantly greater in cells impaled after 120 min compared to cells impaled within the first 30 min. Note that in Ca the value of the fast IJP was 0.0 ± 0.0mV *(* $P<0.05$, $n=5$).*

component reduced from -8.4±0.7mV to -2.6±1.0mV, P<0.005, n=6, Fig 3.4A). 2-APB (50μM) also reduced the amplitudes of the ATP evoked hyperpolarisations (fast component reduced from -13.1±1.3mV to -0.3±0.3mV, slow component reduced from -8.1±0.4 to -0.3±1.1mV, P<0.005, n=4 Fig 3.4B). I also tested whether the direct ATP-evoked response, recorded in TTX (0.6μM) was affected by carbenoxolone. Carbenoxolone significantly reduced the amplitude of the persistent ATP-evoked hyperpolarisation from -4.8±0.6mV to 0.2±1.0mV, n=4 P<0.01, Fig 3.5B). I confirmed that the TTX-insensitive hyperpolarisation due to activation of P2Y1 receptors: it was significantly reduced by MRS2179 (10μM), the P2Y1 anatagonist (Wang et al., 2007, Gallego et al., 2006), from -6.3±0.5 to -4.3±0.8 mV (n=6, P<0.05, Fig 3.5C.)

In response to local exogenous ATP, a few colonic smooth muscle cells showed the biphasic response, similar to those in ileal cells (4 of 50 coupled cells tested), the remaining 46 cells generated only a slow hyperpolarisation (n=8, Fig 3.6A). For colonic smooth muscle cells, hyperpolarisations averaged -10.1±0.7mV in control and did not significantly change with 0.6μM TTX (-9.3±1.0mV, n=4, Fig 3.6B). These results suggest ATP did not activate colonic inhibitory motor neurons as effectively as in the ileum. ATP evoked responses were negligible in circular muscle cells within 30 minutes following dissection of preparations of colon (0.0±0.0mV). Responses significantly increased over 120 minutes (P<0.001, n=4, Fig 3.6C) and stabilised after this. Amplitudes averaged -12.3±1.1mV after 120 minutes. ATP evoked hyperpolarisations in circular muscle of colon, after full responses had developed, were reduced from -12.0±1.3 to -2.4±0.8mV by carbenoxolone (P<0.005, n=4, Fig 3.6E).

3.3.4 Responses to sodium nitroprusside and bethanacol

The effects of NO donor, sodium nitroprusside and bethanacol (exogenous muscarinic agonist) were tested. In responsive cells, sodium nitroprusside failed to produce responses when applied by pressure ejection. Responses to bethanacol varied with each application and therefore could not be used for the purposes of these experiments.

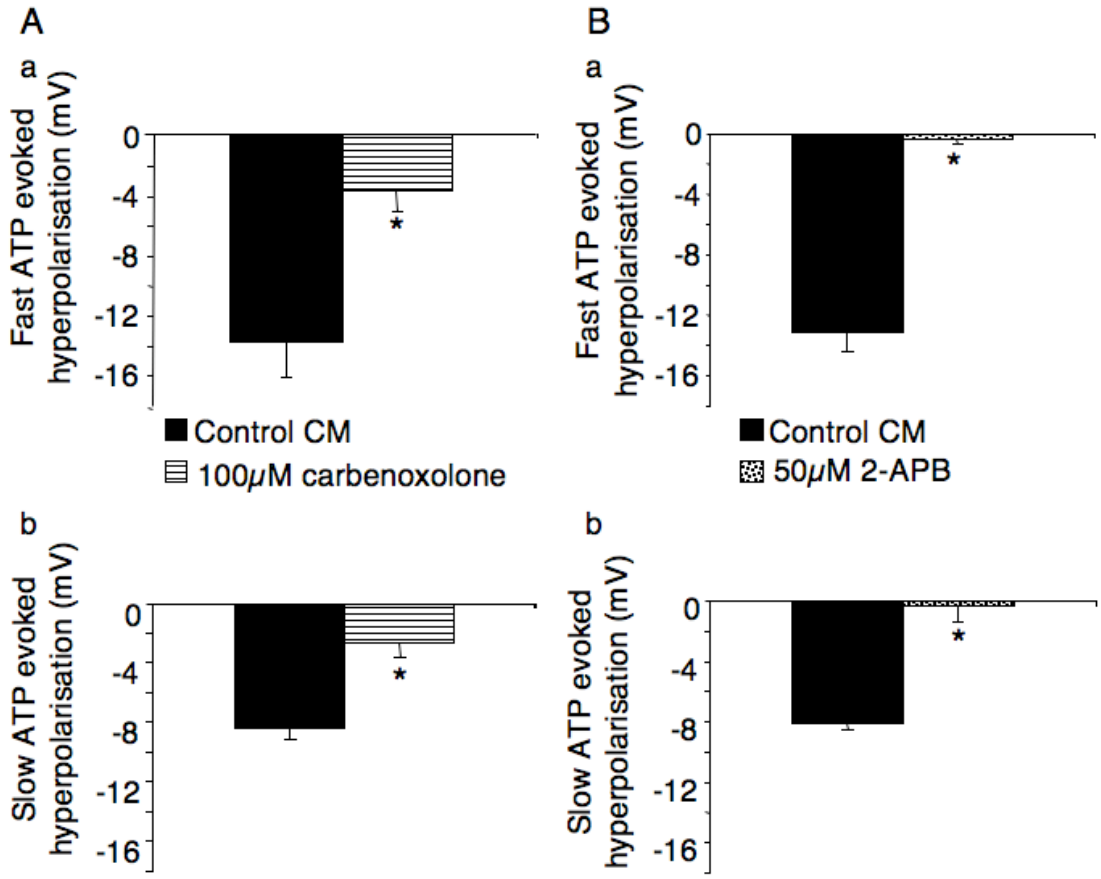


Figure 3.4: The amplitudes of ATP evoked hyperpolarisations were significantly reduced in the presence of 100μM carbenoxolone (Aa fast response, Ab slow response * $P<0.005$, $n=6$) and in 50μM 2-APB (Ba fast response, Bb slow response * $P<0.005$, $n=4$).

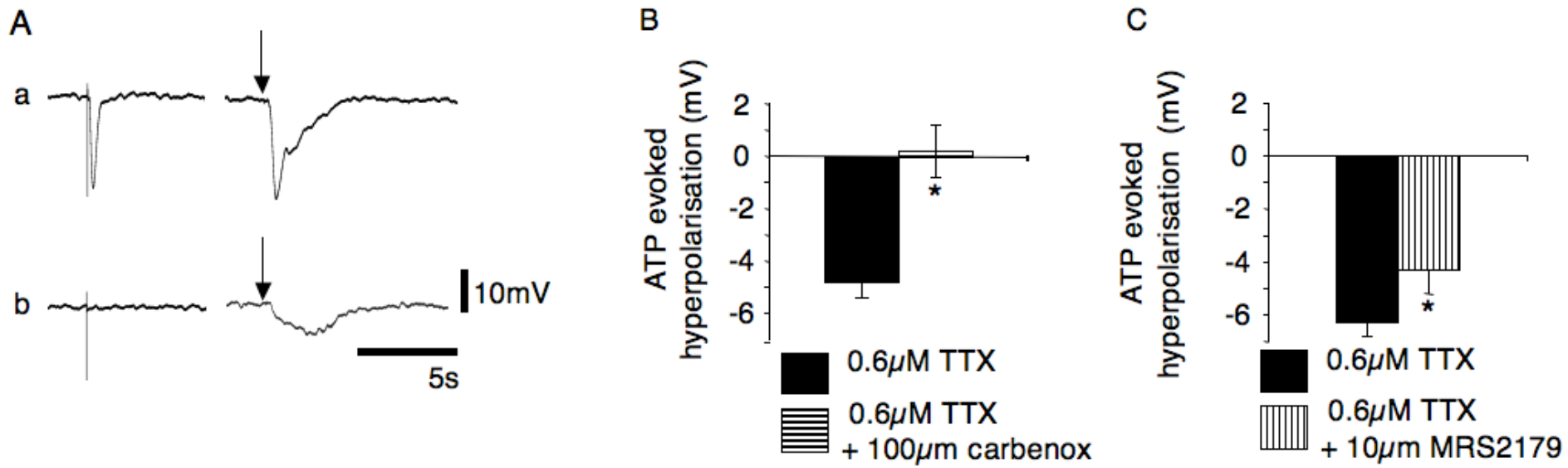
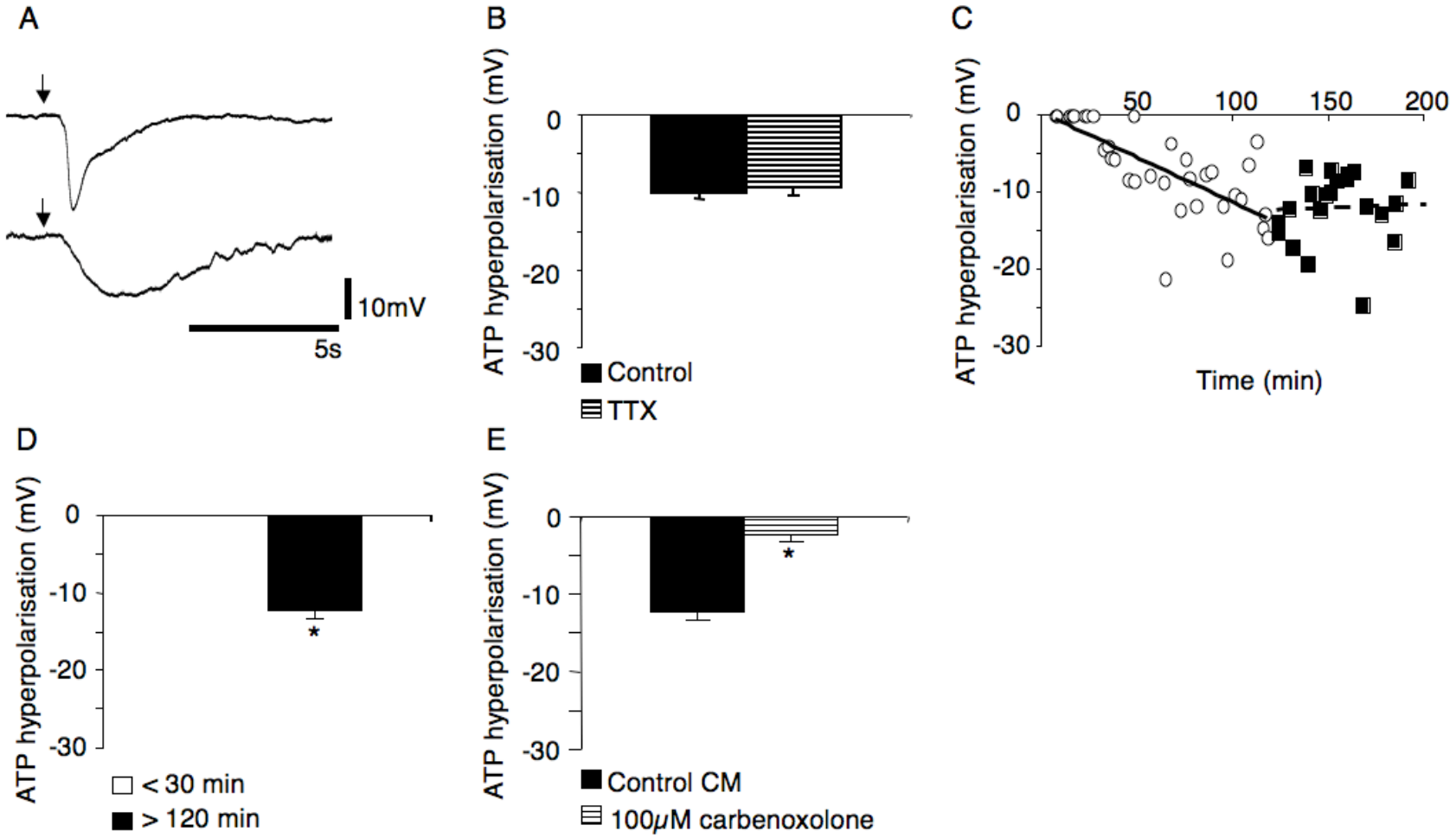


Figure 3.5: The fast ATP-evoked hyperpolarisation in ileal smooth muscle cells was sensitive to TTX (0.6μM). Aa) IJP response (left) and ATP evoked response (right, application at closed arrow) during control conditions. Ab) The fIJP and the fast ATP evoked hyperpolarisations were blocked by 0.6μM TTX. The slow ATP evoked hyperpolarisation remained. B) ATP evoked hyperpolarisations in TTX were significantly reduced with subsequent addition of 100μM carbenoxolone (n=4, P<0.01). C) 10μM MRS2179 significantly reduced the amplitude of TTX-insensitive ATP evoked hyperpolarization (n=6, P<0.05).


A
10mV
5s
B
ATP hyperpolarisation (mV)
0
-10
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150
200
0
-10
-20
-30
Time (min)
D
ATP hyperpolarisation (mV)
0
-10
-20
-30
*
< 30 min
> 120 min
E
ATP hyperpolarisation (mV)
0
-10
-20
-30
*
Control CM
100μM carbenoxolone

Figure 3.6: Exogenous ATP evokes a TTX insensitive hyperpolarisation in colonic smooth muscle cells. A) Application of ATP (10mM) directly onto an impaled smooth muscle cell resulted in a biphasic response in ileal cells (top) versus a uniphasic response in colonic cells (bottom). B) ATP evoked hyperpolarisations did not differ in the presence of TTX (0.6μM, *n*=4). C) Hyperpolarisations to ATP significantly increased in amplitude with time up to 120 minutes following dissection (white dots, *P* <0.0001, *n*=4). Responses did not significantly change beyond 120 minutes (black squares, *n*=4). D) Responses within 30 minutes of dissection were negligible and were significantly greater after 120 minutes (* *P*<0.005, *n*=4). E) The amplitude of ATP evoked hyperpolarisations was reduced by carbenoxolone (* *P*<0.005, *n*=4.)

3.3.5 Visualising dye fills in sectioned preparations

Control preparations of ileum were fixed after fills of circular muscle cells. Preparations were sectioned along either the longitudinal or circumferential axis after different stages following dissection. Of multiple sections, from 26 preparations, of 4 specimens labelling of cells other than circular smooth muscle cells was not obvious. Circumferential sections were cut in parallel with the circular muscle cells allowing the labelled smooth muscle cell to be visualised. Dye coupling as is seen *in situ* was difficult to visualise and could not be quantified. An individual filled smooth muscle cell, or clustered group of cells was seen in 7 of 12 preparations as a single filled strip (Figure 3.7Aa). In the remaining 5 preparations, parallel to the brightly labelled strip, another 1-2 additional smooth muscle profiles were visible (Figure 3.7Ab). These labelled cells did not appear to contact each other. Profiles were 254.9±57.4 µm long and 3.8±0.4µM wide. This is smaller than dimensions from cell labelling counted *in situ* since preparations were dehydrated. Longitudinal sections were cut perpendicular to the axis of dye fills. Dye fills appeared as a small line 15.1±2.4µm long and 4.0±0.6µm wide. Other labelled structures were not visualised in these preparations (Figure 3.7B).

3.3.6 Recording from a cell other than circular muscle cell

While attempting to impale circular muscle cells figure 3.8A demonstrates another cell type that was also recorded from. It did not have the appearance of a smooth muscle cell, however a -13.3mV fIJP was recorded from this cell, until the impalement was prematurely lost (Fig 3.8B). The impalement was not long enough for membrane potential to stabilise or for input resistance to be calculated. The cell was dye filled so that its structure could be visualised. A cell body and multipolar globular processes could be visualised by the dye fill; dye coupling was not obvious (Figure 3.8A). This was the only occasion that I am aware of that a cell with this structure was recorded from. Immunohistochemistry was attempted on this preparation to identify the cell, but carboxyfluorescein labelling faded following the procedure. However, the

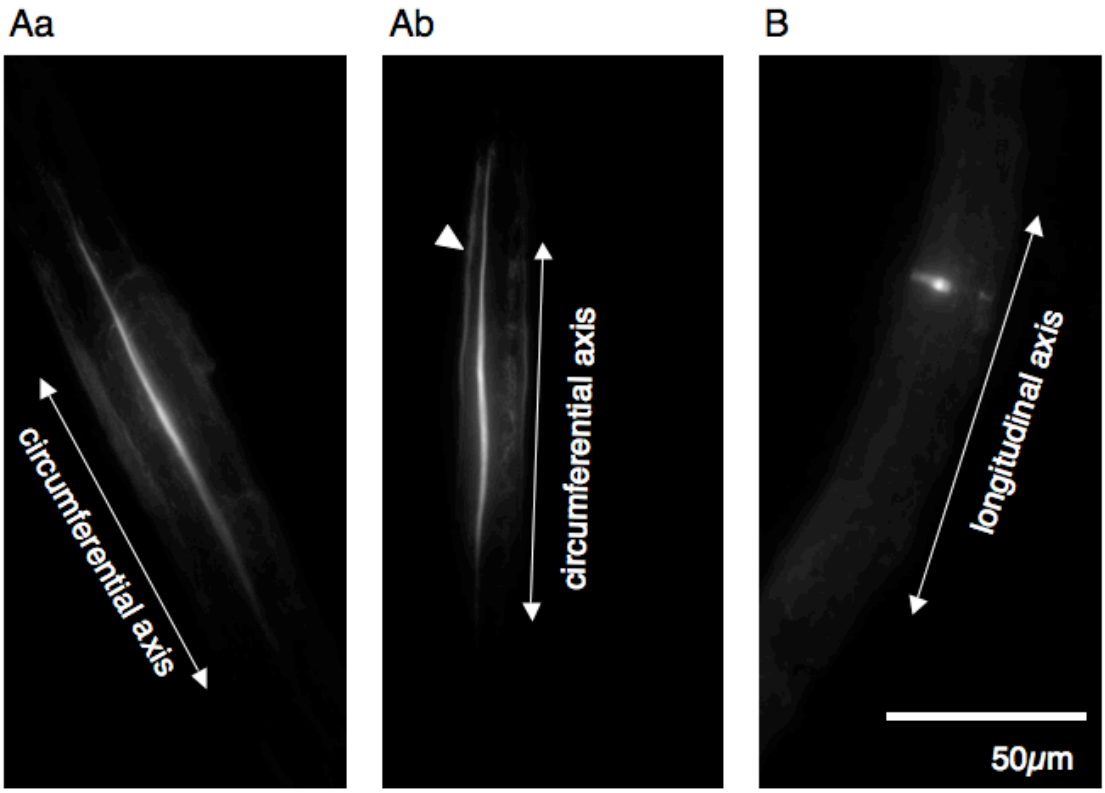

Figure 3.7: Sections of preparations with carboxyfluorescein filled circular muscle cells. Preparations were cut with the circumferential (A) or longitudinal (B) axis. Aa) An individual cell/group of cells were labelled and visualised as a single strip. Ab) In 5 of 12 preparations, additional cells were labelled adjacent to the brightly filled cell (arrow head). B) A brightly filled spot or strip was seen in longitudinal sections. Cells appear smaller than expected since preparations were not re-hydrated after sectioning.

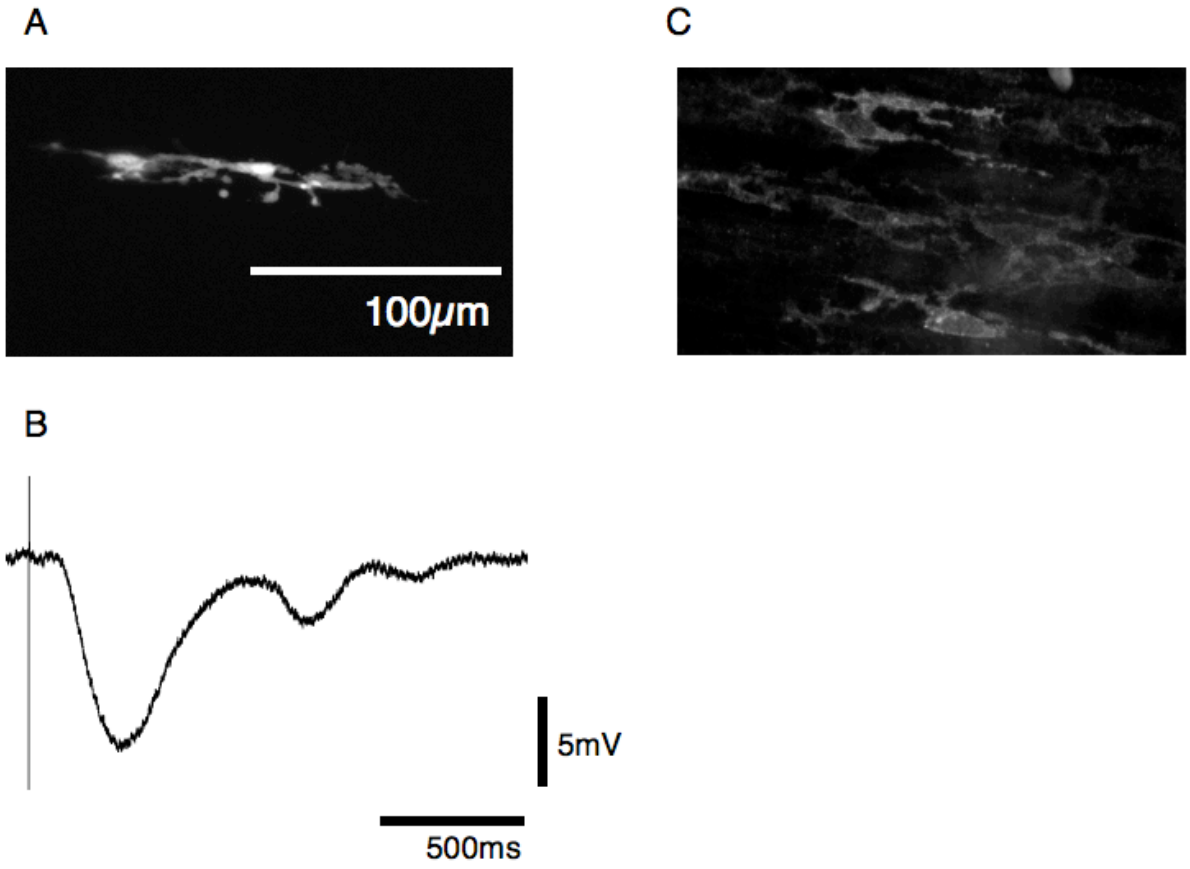

Figure 3.8: Partially filled cell with different morphology to a smooth muscle cell (A). A fIJP was recorded from this cell before the impalement was lost (B). The structure of the labelled cell resembled that of fibroblast-like cells immunoreactive for PDGFRα (C).

structure of the cell resembled that of fibroblast-like cells immunoreactive for platelet derived growth factor receptor alpha (Figure 3.8C, *n*=3).

3.3.7 Spontaneous contractions following inhibition of gap junctions

Spontaneous contractions were measured after 120 minutes recovery from dissection before control data and application of drugs was trialled. Basal tone, amplitude of spontaneous contractions and area under the curve (AUC) were not significantly different for specimens with the mucosa attached compared to specimens with the mucosa removed (*n*=4). In preparations with the mucosa intact basal tone did not change following addition of carbenoxolone or TTX (Fig 3.9A, 3.10A). The amplitude of spontaneous contractions averaged 0.6±0.2g in control. Addition of carbenoxolone non-significantly reduced contraction amplitude (0.3±0.12g), however with sequential addition of TTX amplitudes were significantly reduced compared to control (0.1±0.0g, $P<0.05$, *n*=4, Fig 3.10B). AUC for preparations with intact mucosa averaged 6.0±2.5gs in control and 3.0±1.5gs in carbenoxolone. With sequential addition of TTX, AUC was significantly smaller compared to control (0.6±0.2gs, $P<0.01$, *n*=4, Fig 3.10C). For preparations without the mucosa, compared to control amplitudes (1.0±0.3g) spontaneous contractions reduced with addition of carbenoxolone (0.5±0.2g, $P<0.05$, *n*=4) and TTX (0.04±0.01g, $P<0.001$, *n*=4, Fig 3.9B, 3.10D). Addition of carbenoxolone to mucosa-free preparations significantly reduced the AUC of contractions from 9.8±3.0gs to 4.2±1.6gs ($P<0.05$, *n*=4, Fig 3.10F) and sequential addition of TTX amplitudes were reduced compared to control 0.5±0.0gs ($P<0.001$, *n*=4, Fig 3.10F).

3.4 Discussion

Pharmacological blockade of gap junctions with carbenoxolone, 18ß glycyrrhetinic acid and 2-APB returned fully responsive circular muscle cells to an unresponsive state similar to that following initial dissection and set up. The amplitude of junction potentials was reduced, RMP hyperpolarised, input resistance increased and dye coupling decreased. Responses to exogenous ATP were initially suppressed from the

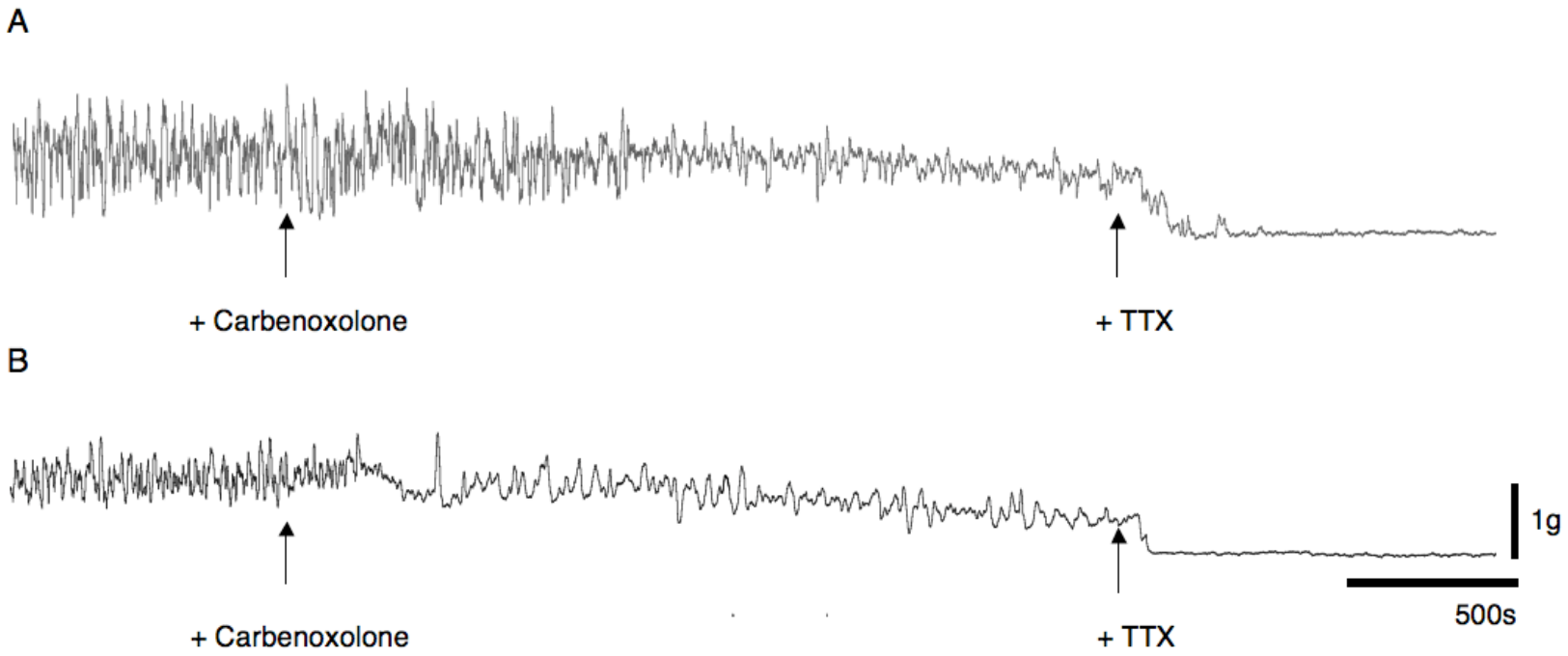

Figure 3.9: The force of colonic circular contractions with addition of carbenoxolone and TTX (arrows) for preparations with the mucosa intact (A) or removed (B).

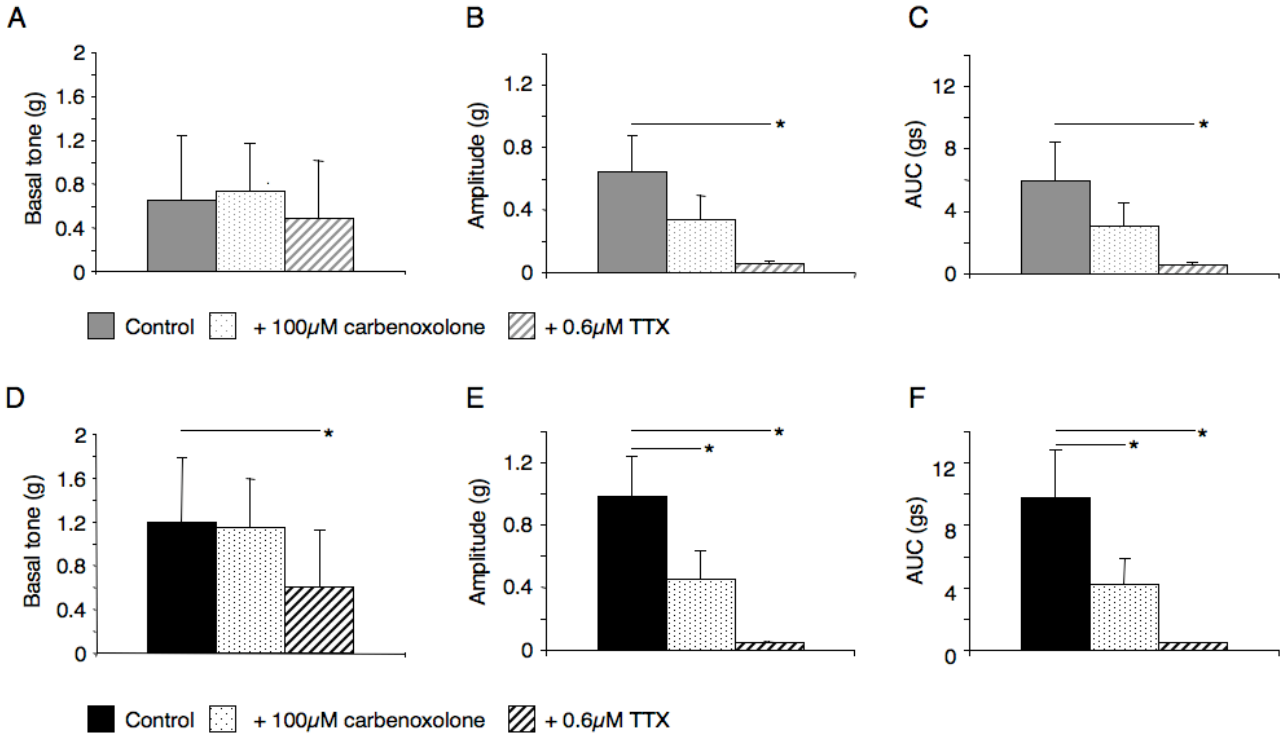

Figure 3.10: For preparations with the mucosa intact (grey), basal tone did not significantly change with addition of carbenoxolone or TTX (A, n=4). The amplitude and AUC of spontaneous contractions significantly decreased compared to control with sequential addition of TTX following carbenoxolone (B and C respectively, P<0.05, n=4). In preparations with the mucosa removed (black), basal tone significantly decreased with sequential addition of TTX compared to control (D, P<0.05, n=4). The amplitude and AUC of spontaneous contractions (E and F respectively) significantly decreased compared to control following addition of carbenoxolone (P<0.05), then TTX (P<0.01, n=4).

moment when preparations were set up and recovered over the following 120 minutes. This demonstrates that a post junctional- mechanism probably contributes to the suppression of the fast IJP following initial set up. This suggests that modulation of intercellular coupling between smooth muscle cells can potently modify their physiology. This potentially represents a novel mechanism that may influence smooth muscle physiological responses. The finding that all gap junction blockers caused significant decreases in dye coupling between circular smooth muscle cells, as measured by both dye-injection and current-injection protocols suggests that the intracellular dye injection method used here was a reliable method to measure gap-junction coupling between smooth muscle cells. Similar methods have been used by other authors, to demonstrate gap-junction coupling between enteric glial cells (Hanani et al., 1989, Maudlej and Hanani, 1992), between ICC (Belzer et al., 2002, Belzer et al., 2004) and between gut smooth muscle cells (Farraway et al., 1995, Zamir and Hanani, 1990).

3.4.1 Gap junction uncoupling and the loss of junction potentials

It is interesting to speculate on how changes in gap junction coupling may be related to the loss of junction potentials and responses to exogenous ATP. One possible explanation is that loss of coupling blocks the spread of junction potentials from a subset of smooth muscle cells that directly receive input from motor neuron axons ("responder cells"), to other cells. This seems unlikely. I recorded from over 100 uncoupled smooth muscle cells during the study and none had intact (full amplitude) junction potentials or responses to ATP in gap junction blockers or in the first 30 minutes after dissection. Either "responder" cells are very rare, or motor neurons transmit via another type of cell that I did not record from, and which is coupled to smooth muscle cells. This latter explanation is compatible with my observation that responses to exogenous ATP were also blocked in all uncoupled smooth muscle cells, suggesting that smooth muscle cells lack sufficient purine receptors and/or ion channels to support a full IJP.

3.4.2 Interstitial Cells of Cajal and neuromuscular transmission

One possibility is that motor neurons act via ICCs that are gap junction coupled to circular muscle cells. Immunohistochemistry and electron microscopy have demonstrated that in the guinea pig ileum ICC of the deep muscular plexus (ICC-DMP) are closely associated with axons of inhibitory and excitatory motor neurons, identified by nitric oxide synthase and substance P immunoreactivity, respectively (Wang et al., 1999). Nitrergic junction potentials in smooth muscle cells of the murine stomach are mediated indirectly, at least in part by nitric oxide acting on intramuscular ICC (ICC-IM), which are coupled to smooth muscle cells (Suzuki et al., 2003b, Burns et al., 1996). In W/W^v mice, which lack gastric ICC-IM, nitrergic hyperpolarisations are significantly reduced in amplitude. Similarly, electrically stimulated EJPs were negligible in gastric smooth muscle cells from W/W^v mice (Ward et al., 2000c). In the present study, slow IJPs, which are largely nitrergic (Goyal and He, 1998, Lyster et al., 1992), and EJPs were suppressed during the first 30 minutes. I speculate that this may have been due to loss of coupling between smooth muscle cells and the ICC-DMP that mediate them. However, purinergic transmission is probably not mediated via ICCs, since apamin-sensitive purinergic IJPs persist in W/W^v mice (Suzuki et al., 2003b, Burns et al., 1996). Immunohistochemical studies in the human colon have demonstrated that Kit-immunoreactive cells are not immunoreactive for SK3 (Vanderwinden et al., 2002a).An alternative explanation is needed to account for the loss of purinergic fast IJPs during the first 30 minutes.

3.4.3 Fibroblast-like cells and neuromuscular transmission

"Fibroblast-like cells" have been suggested to play a role in smooth muscle responses to purines released from axons of inhibitory motor neurons. The purinergic fast IJP is primarily mediated by opening of SK3, small conductance, calcium-dependent potassium channels (Wang et al., 2007, Klemm and Lang, 2002) and is blocked by the bee venom, apamin (Bauer and Kuriyama, 1982b). Fibroblast-like cells in the guinea pig colon are immunoreactive for SK3 channels (Klemm and Lang, 2002). Electron microscopy has demonstrated gap junctions between fibroblast-like cells and outer circular muscle cells; they also receive close contacts

from motor neuron axons (Zhou and Komuro, 1992). Fibroblast-like cells are not immunoreacitve for c-kit, but are specifically labelled by antisera to PDGFRα (platelet-derived growth factor receptor α) (Iino et al., 2009) and CD34 and SK3 channels (Vanderwinden et al., 2002a), suggesting that they are not ICCs. Isolated "fibroblast-like cells" from the mouse colon generate large amplitude outward currents in response to ATP which are inhibited by apamin and the $P2Y_1$ receptor antagonist MRS2500 (Wang et al., 2007, Klemm and Lang, 2002). Current density in smooth muscle cells exposed to exogenous ATP was 1-2% of that in fibroblast-like cells (Kurahashi et al., 2011).

I speculate that purinergic responses in guinea pig ileum are generated primarily in fibroblast-like cells and then spread via gap junctions into the circular smooth muscle (Kurahashi et al., 2011). This would readily explain why a reduction in gap-junction coupling in the first 30 minutes might be closely associated with temporary suppression of fIJPs, and with a parallel loss of responses to exogenous ATP. When ATP was pressure ejected onto the recorded cell, it covered an area approximately 200-400μm wide, which would include both ICC and fibroblast-like cells. It should be noted that the routine use of carboxyfluorescein in recording electrodes indicated that fibroblast-like cells were very rarely impaled during this study. In one instance, one cell with morphology distinct from smooth muscle cells was labelled and a fIJP was recorded from this cell. Unfortunately immunohistochemical analysis of this preparation was not possible, however, the cell had multipolar, globular processes, and resembled fibroblast-like cells that are immunoreactive for PDGFRα (Iino and Nojyo, 2009, Kurahashi et al., 2012, Kurahashi et al., 2011). This highlights the difficulty of targeting fibroblast-like cells for intracellular electrophysiology from wholemount preparations. Methods using transgenic mice expressing enhanced green fluorescent protein in these cells maybe the best option so that their exact location can be highlighted and they can be targeted for recording (Kurahashi et al., 2011).

One observation however suggests that intermediate cells may not be involved in neuromuscular transmission. In none of the wholemount or sections of dye fills were

either ICCs or fibroblast-like cells seen to be dye coupled to smooth muscle cells. The reasons for this are not clear. It is possible that gap junctions between pairs of smooth muscle cells differ from those that connect smooth muscle cells to ICCs or to fibroblast-like cells (Seki and Komuro, 2001). Alternatively, coupling between ICC and smooth muscle may be scarce. It should also be noted that in a previous study, in which interconnected ICCs were intracellularly filled with dye in guinea pig small intestine, coupled smooth muscle cells were not labelled (Belzer et al., 2002, Belzer et al., 2004).

3.4.4 Gap junction coupling and spontaneous contractions

In the colon, carbenoxolone limited the amplitude and area under the curve of spontaneous contractions. Residual contractions were almost abolished by TTX. Since TTX inhibited residual contractions, it suggests that some neuromuscular transmission persists in the presence of blockers and this is supported by the electrophysiological data, where the amplitude of fIJPs are reduced by gap junction blockers but are not ablated. One can speculate that contractions driven by pacemaker cells were inhibited since spontaneous activity is lost with carbenoxolone and TTX, however these experiments would need to be repeated by applying the drugs in reverse order to confirm this. Previous studies in canine colon in the presence of TTX have demonstrated that the amplitude of spontaneous contractions was significantly reduced by low concentrations of carbenoxolone. At higher concentrations the frequency was also reduced (Daniel et al., 2001).

3.4.5 The specificity of gap junction blockers

A specific and selective small molecular inhibitor of gap junctions has yet to be identified (Rozental et al., 2001). The gap junction blockers used in this study have actions at other sites. Glycyrrhetinic acid can inhibit voltage sensitive Ca2+ currents; carbenoxolone (a type of glycyrrhetinic acid) additionally inhibits 11ß-hydroxysteroid hydrogenase the enzyme which converts inert cortisol to active cortisol; and 2-APB blocks intracellular Ca2+ signaling by inhibiting IP3 induced

Ca2+ release and can stimulate TRPV1 receptors (Juszczak and Swiergiel, 2009). Potentially, these off-target-effects raise doubts about the interpretation of findings in this chapter. However, these side effects cannot explain our observations. I used 3 different gap junction blockers, with different mechanisms of action all of which mimicked the uncoupling that occurred at the start of recovery period and corresponding electrophysiological changes. Post junctional responses and spontaneous contractions was also significantly diminished with the gap junction blockers tested, however small responses still remained. This may be explained by the fact that the agents used in this study do not 100% block gap junction conductance (Rozental et al., 2001, Bai et al., 2006). Carbenoxolone and 18ß glycyrrhetinic acid reduce gap junctional conductance by around 80% and 60% (Rozental et al., 2001). The IC_{50} of 2-APB varies depending on the connexin the channel is comprised of, for Cx 43 this is ~51μM while for Cx45 it is ~18μM (Bai et al., 2006). Agents such as heptanol and octanol result in complete gap junction blockade however their mechanism of action is thought to be by changing the fluidity of the cell's membrane therefore these agents also affect other ion channels and have their own complications. Connexin mimetic peptides are synthetic peptides, which specifically inhibit gap junctions, however their efficacy does not exceed that of the agents tested (Rozental et al., 2001). They were not tested here due to their slow onset of action, and their cost for the protracted recording period of these studies.

The present study showed that the initial suppression of gap-junction coupling occurs in circular smooth muscle cells of guinea pig ileum and colon. Changes in coupling may not affect inhibitory neuromuscular transmission equivalently in all species and regions of gut. IJP amplitude was reduced in circular muscle cells of murine proximal colon in the presence of heptanol and octanol, but unaffected by carbenoxolone or the connexin 43 blocking peptide, GAP-27 (Sibaev et al., 2006). Similar findings have been reported for murine small intestine (Daniel et al., 2007). Gap junction permeability were not directly assessed in these studies and I speculate that the concentration of GAP-27 used was not high enough (500μM) for maximal

blockade of coupling (up to10mM.) However, it is also possible that there are differences in gap junction pharmacology between species or regions of gut.

3.4.6 Exogenous ATP activates inhibitory motorneurons in the ileum but not the colon

Another interesting result from this study was that responses of colonic smooth muscle cells to exogenous ATP were TTX-insensitive compared to ileal smooth muscle which had TTX-sensitive and insensitive components. This result suggests that in the ileum, ATP was activating inhibitory motor neurons generating a fIJP in the smooth muscle cell. This is consistent with intracellular recordings from myenteric neurons in ileum and colon of the guinea pig, where purinergic fast excitatory post synaptic potentials (fEPSPs) are more prominent in the ileum than the distal colon (LePard et al., 1997, Nurgali et al., 2003). A small subset of fast EPSPs in myenteric neurons is due to activation of P2X receptor subtypes (Zhou and Galligan, 1996). In the ileum there is some suggestion that purinergic fEPSPs are specific to descending neurons (Johnson et al., 1999) however a role for P2X receptors has been demonstrated in neuro-neuronal purinergic transmission not only in the descending inhibitory pathways (Bian et al., 2000a) but also in the ascending pathways (Spencer et al., 2000). Both ascending and descending myenteric neurons receive fEPSPs in the guinea pig colon (Nurgali et al., 2003). The percentage of myenteric neurons immunoreactive for nitric oxide synthase (NOS) that are also immunoreactive for $P2X_2$ was 91% in the guinea pig ileum and 96% in the distal colon (Castelucci et al., 2002). Of myenteric neurons immunoreactive for NOS, 65% and 80% were immunoreactive for $P2X_3$ in the ileum and distal colon (Poole et al., 2002).Therefore an explanation for the activation of a TTX sensitive hyperpolarisation in ileal compared to colonic smooth muscle cells may be due to a greater number of inhibitory motorneurons with P2X receptors or a higher concentration of P2X receptors on ileal inhibitory motorneurons compared to colonic. It is also worth considering evidence that transmission between descending interneurons in the guinea pig ileum, may involve purinergic transmission. Electrical stimulation of the mucosa, or distension applied oral to a recorded neuron evokes slow and intermediate EPSPs that are inhibited by P2Y1 antagonist MRS 2179

(Gwynne and Bornstein, 2009, Thornton et al., 2013). Given how close to the recording electrode ATP was applied, and the short delay between ATP application and the fast-ATP evoked response it seems unlikely that this neuro-neurotransmission would be accounting for the response, however, it cannot be ruled out.

3.5 Conclusion

Pharmacological blockade of gap junctions caused similar changes to those occurring in the first 30 minutes of recording. Responses to exogenous ATP were ablated within 30 minutes following dissection and recovered within 120 minutes, in the same manner as fIJPs. This suggests that a post-junctional change is responsible for the loss of junction potentials. I have demonstrated that changes in gap junction coupling have a profound effect on smooth muscle responses and are subject to modulation. However, the mechanism by which uncoupling takes place while tissues are being prepared for recording remains to be determined. Nevertheless, if such mechanisms operate under physiological conditions, they could represent a novel mechanism in the control of gut motility.