Gap junction coupling between smooth muscle cells modulates responses to inhibitory motorneurons and exogenous ATP

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We have previously reported that electrophysiological responses of gut smooth muscle cells, including inhibitory junction potentials, are absent during the first 30-60 minutes after setting up preparations *in vitro*. Here, we investigated the mechanisms that underlie this temporary unresponsiveness.

Methods: Segments of guinea pig ileum, with mucosa and submucosa removed, were used for intracellular recording under current clamp conditions. Circular smooth muscle cells were impaled with glass micropipettes filled with carboxyfluorescein (5%) and KCl (1M), in Krebs solution (34°C) containing 1μM hyoscine and 1μM nicardipine to inhibit smooth muscle contractions. We recorded resting membrane potential (RMP), inhibitory junction potentials (IJPs) evoked by single shot stimuli, and responses to ATP applied locally by pressure ejection (140kPa nitrogen pulses, 50-100ms duration, 10mM). Cells were dye-filled by 0.5nA hyperpolarising pulses (50% duty cycle for 2 minutes, followed by 1 minute diffusion). Dissections were carried out in cool Krebs solution (14°C). The start of the equilibration period was considered as being the moment when warmed Krebs solution (35°C) first reached the recording chamber.

Results: The recording chamber warmed to 34-35°C within the first 5 minutes of the equilibration period (n=3), however IJP amplitude was typically less than 1mV for the first 30 minutes (n=12). IJP amplitude then increased gradually so that by 90-120 minutes, IJP amplitude averaged -11.5mV±1.6mV (n=12). Cells with IJPs less than 1mV were classed as 'unequilibrated' and cells with IJPs greater than 10mV were classed as 'equilibrated'. 'Unequilibrated' cells were significantly hyperpolarised compared to equilibrated cells where RMPs were -58.8±1.4mV and -47.2±0.4mV respectively (cells=23 and 64, n=12, $p<0.0001$). Input resistance was significantly greater in 'unequilibrated' cells (15.5±1.9 MΩ) than 'equilibrated' cells (8.7±0.7 MΩ, n=12, $p<0.0001$). 'Unequilibrated' cells showed significantly less dye coupling (mean=2.1±0.3 carboxyfluorescein-filled profiles) compared to 'equilibrated' cells (mean=4.4±0.3, n=12, $p<0.0001$). Addition of gap junction blockers carbenoxolone (100μM) and 18β Glycyrrhetinic acid (10μM) made "equilibrated" cells change their characteristics back to a state similar to "unequilibrated" cells. In the presence of carbenoxolone, circular smooth muscle cells had a more negative resting membrane potential, smaller IJP amplitude, increased input resistance and reduced dye coupling. Glycyrrhetinic acid similarly reduced IJP amplitude, increased input resistance and reduced dye coupling between 'equilibrated' cells. These results suggests that gap junction coupling between smooth muscle cells may increase during the equilibration period and may underlie the changes in IJP amplitude. In other cell types, gap junction permeability is reduced by high cytostolic [Ca^{2+}]. We tested whether influx of calcium, during the set-up procedure, might cause uncoupling during the 'equilibration' period. Preparations were dissected in low [Ca^{2+}], high [Mg^{2+}] Krebs solution (mM: Ca^{2+} 0.25; Mg^{2+} 2.5 at 14°C), and transferred into normal Krebs solution at 35°C for recording. There was no significant reduction of the equilbration period in these preparations. Lastly we tested whether the loss of IJPs during the equilibration period was associated with a loss of sensitivity by smooth muscle cells to ATP; the transmitter of the fast IJP. Hyperpolarisations evoked by local application of 10mM ATP were significantly smaller in 'unequlibrated'cells (0.3±0.3mV) compared to 'equilibrated' cells (-13.0±1.1mV, n=6, $p<0.0001$)), paralleling the change in amplitude of the IJP. This suggests that the loss of neuronal input may be due to a change in electrophysiological properties of the postsynaptic cell during equilibration.

In conclusion, following set-up of dissected gut preparations *in vitro*, smooth muscle cells show significant changes in electrophysiological properties that recover during the "equilibration period". Smooth muscle cells are hyperpolarised and have a reduced response to inhibitory stimulation either by inhibitory motorneurons or by exogenous application of ATP. During this period gap junction coupling is suppressed. Pharmacologically blocking gap junctions mimics the change in electrophysiological properties during the equilibration period. The change in gap junction coupling does not appear to be due to influx of calcium into smooth muscle cells during the set-up procedure.