Microdomains of muscarinic acetylcholine and Ins(1,4,5)P₃ receptors create ‘Ins(1,4,5)P₃ junctions’ and sites of Ca²⁺ wave initiation in smooth muscle

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Summary
Increases in cytosolic Ca²⁺ concentration ([Ca²⁺]c) mediated by inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃, hereafter InsP₃] regulate activities that include division, contraction and cell death. InsP₃-evoked Ca²⁺ release often begins at a single site, then regeneratively propagates through the cell as a Ca²⁺ wave. The Ca²⁺ wave consistently begins at the same site on successive activations. Here, we address the mechanisms that determine the Ca²⁺ wave initiation site in intestinal smooth muscle cells. Neither an increased sensitivity of InsP₃ receptors (InsP₃R) to InsP₃ nor regional clustering of muscarinic receptors (mAChR3) or InsP₃R1 explained the selection of an initiation site. However, examination of the overlap of mAChR3 and InsP₃R1 localisation, by centre of mass analysis, revealed that there was a small percentage (~10%) of sites that showed colocalisation. Indeed, the extent of colocalisation was greatest at the Ca²⁺ wave initiation site. The initiation site might arise from a selective delivery of InsP₃ from mAChR3 activity to particular InsP₃R to generate faster local [Ca²⁺]c increases at sites of colocalisation. In support of this hypothesis, a localised subthreshold ‘priming’ InsP₃ concentration applied rapidly, but at regions distant from the initiation site, shifted the wave to the site of the priming. Conversely, when the Ca²⁺ rise at the initiation site was rapidly and selectively attenuated, the Ca²⁺ wave again shifted and initiated at a new site. These results indicate that Ca²⁺ waves initiate where there is a structural and functional coupling of mAChR3 and InsP₃R1, which generates junctions in which InsP₃ acts as a highly localised signal by being rapidly and selectively delivered to InsP₃R1.

Key words: Ca²⁺ waves, InsP₃, Smooth muscle

Introduction
Activities which include blood flow through vascular blood vessels, peristaltic motion in the gastrointestinal tract and rhythmic contractions of the uterus during labour are all regulated by contraction of smooth muscle. Transient increases in the cytosolic Ca²⁺ concentration ([Ca²⁺]c) provide the trigger for contraction but also affect other different, at times contradictory, processes including cell growth, division and apoptosis. Ca²⁺ may regulate so many complex behaviours at least in part by the cell restricting the location of increases in [Ca²⁺]c to specific sites in the cell by the distribution of various Ca²⁺ signalling proteins. Under certain (as yet ill-defined) conditions, the Ca²⁺ signal may be propagated through the cell to extend the reach of the signal and enable Ca²⁺ to encode for many cellular processes.

[Ca²⁺]c increases occur via Ca²⁺ influx through voltage-gated and receptor-operated Ca²⁺ channels on the plasma membrane or release from inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃, hereafter InsP₃] receptors (InsP₃R) and ryanodine receptors (RyR) present on the sarcoplasmic reticulum (SR), an internal Ca²⁺ store (Chalmers et al., 2007). The generation of an InsP₃-mediated Ca²⁺ signal occurs via extracellular activation (e.g. agonists) of G protein coupled receptors present on the plasma membrane. Activation leads to phospholipase C β (PLC-β) catalysed formation of InsP₃, a diffusible messenger that, with Ca²⁺, activates InsP₃R. Interestingly, although a rapidly diffusing (diffusion coefficient=300 μm² s⁻¹) messenger presumably with free access throughout the cell, InsP₃ may in some circumstances act as a local rather than global signal to generate specificity in receptor function. Some explanation for the local nature of the signal is found in the observation that certain receptors colocalize with InsP₃R to form a local signalling complex (Delmas et al., 2002; Hur et al., 2005; Lur et al., 2011; Yuan et al., 2005). For example, while muscarinic and bradykinin receptors each stimulate PLC, only bradykinin receptors co-immunoprecipitate with, and activate, InsP₃R to evoke Ca²⁺ release (Delmas et al., 2002). The arrangement enables PLC activation by muscarinic and bradykinin receptors to evoke different cellular responses. The positioning of active InsP₃R near the plasma membrane provides a mechanism to facilitate agonist activation, acting via InsP₃, to target specific types of cellular response, i.e. by generating Ca²⁺ rises in specific regions of the cell (Smith et al., 2009). Additional local specificity is achieved by clustering surface receptors in certain regions on the plasma membrane, which gives rise to areas with increased sensitivity to extracellular stimuli (Thomason et al., 2002). Together the clustering of surface receptors and subplasma membrane location of InsP₃R permits agonists that activate G protein coupled receptors to generate differences in which signalling pathways are subsequently activated and direct,
specific associations between signalling proteins located at the plasma membrane and the SR membrane to occur (Hur et al., 2005; Yuan et al., 2005). These arrangements enable selective changes in \([\text{Ca}^{2+}]_c\) and cell performance to occur from messengers which are normally thought to evoke activity throughout the cell.

Under some conditions the reach of a local \([\text{Ca}^{2+}]_c\) signal may be selectively extended by the initial, \([\text{Ca}^{2+}]_c\) rise being propagated through the cell. The cell normally retains careful control of the signal reach by regulating \(\text{InsP}_3\) distribution (McCarron et al., 2004). In addition, in some cells with particular arrangements in their gross cell structure, such as hippocampal neurons, exocrine acinar cells and hepatocytes, the \([\text{Ca}^{2+}]_c\) wave initiation site and direction of travel of the wave appears linked to the expression and distribution of \(\text{InsP}_3\) subtype (Hernandez et al., 2007; Jacob et al., 2005; Takemura et al., 1999), presumably to maintain selectivity in responses. For example, although \(\text{InsP}_3\)R1 were expressed throughout the cell, \(\text{InsP}_3\)R-mediated \([\text{Ca}^{2+}]_c\) waves initiated from the apical region where \(\text{InsP}_3\)R2 (hepatocytes) and \(\text{InsP}_3\)R3 (ocular ciliary polarised epithelial cells) were expressed (Hirata et al., 1999; Rooney et al., 1990).

Although many other cell types such as smooth muscle do not display any apparent structural polarity they too have sites where \([\text{Ca}^{2+}]_c\) waves preferentially initiate. The mechanisms which contribute to the sites at which a \([\text{Ca}^{2+}]_c\) wave initiate in these cells are unknown and addressed in the present study. Local differences in the sensitivity to \(\text{InsP}_3\) did not explain the wave initiation site. Nor did the expression patterns of mACHR3 and \(\text{InsP}_3\)R1 indicate any apparent regional receptor clustering. Yet, dual labelling of \(\text{InsP}_3\)R1 and mACHR3 revealed a small percentage of colocalization (\(\sim 10\%)\) of the receptors. This colocalisation was greater at sites where \([\text{Ca}^{2+}]_c\) waves initiated. The wave initiation site may arise from the rapid delivery of \(\text{InsP}_3\) to particular \(\text{InsP}_3\)R. In support, the wave initiation site could be altered either by locally increasing \([\text{InsP}_3]\) at a location distant to the initiation site or by rapidly buffering the \([\text{Ca}^{2+}]_c\) increase at the initiation site. This work demonstrates that the initiation site of a \([\text{Ca}^{2+}]_c\) wave is not dependent on the density or expression of \(\text{InsP}_3\)R present at that location but is dependent on the close proximity of mACHR and \(\text{InsP}_3\)R and rapid delivery of \(\text{InsP}_3\) to particular \(\text{InsP}_3\)R.

**Results**  
**\(\text{InsP}_3\)-mediated \([\text{Ca}^{2+}]_c\) waves initiate at a single location**

In voltage-clamped single colonic smooth muscle cells depolarization (\(-70\) to \(+10\) mV) activated an inward current and transient increase in \([\text{Ca}^{2+}]_c\) (Fig. 1Aa,Ab). The \([\text{Ca}^{2+}]_c\) increase initiated at the same time throughout all regions of the cell presumably due to the concerted opening of voltage-gated \([\text{Ca}^{2+}]_c\) channels across the plasma membrane (McCarron et al., 2008). In the same cell, the muscarinic acetylcholine receptor
agonist carbachol (100 μM), applied by hydrostatic pressure ejection, evoked InsP3-mediated Ca2+ release (Fig. 1Ba,Bb). Carbachol was applied in a Ca2+-free solution so that the Ca2+ response was only due to Ca2+ release from the SR. InsP3R activity is required for the initiation of carbachol-evoked Ca2+ waves. In support, the InsP3R inhibitor 2-aminoethoxydiphenyl borate (2-APB) blocked the carbachol-evoked Ca2+ wave. The [Ca2+]i increased by 1.24±0.22 F/F0 in control and by 0.14±0.04 F/F0 after 2-APB (10 μM) preincubation, respectively (n=5; P<0.01; not shown). Neither RyR activity nor SR store content are affected by 2-APB in this cell type supporting the selectivity of action of 2-APB at InsP3R (McCarron et al., 2002).

Carbachol-evoked Ca2+ release initiated typically at one site (Fig. 1Aa, panel 1, region i) and propagated the length of the cell as a Ca2+ wave (Fig. 1Ba,Bb). Interestingly, subsequent applications of carbachol at ~90 sec intervals evoked Ca2+ release of similar magnitude which initiated from the same location each time. There was no correlation between the location of agonist application and site of Ca2+ wave initiation (data not shown). Nor was the initiation site located preferentially at the nucleus or site of patch electrode attachment (data not shown). In cells that were not voltage-clamped, repetitive applications of carbachol also evoked Ca2+ waves, each of which initiated at the same single site (data not shown). The amplitude of the Ca2+ wave varied in different regions of the cell, often there was greater release near the nucleus (McCarron et al., 2010). The mechanisms that may contribute to the occurrence of an ‘eager’ site of Ca2+ wave initiation were examined first by determining InsP3R and mAChR expression patterns and then their relative functional contributions to wave initiation.

**Distribution of proteins involved in InsP3-mediated Ca2+ release**

The distribution of InsP3R and mAChR was examined by fluorescence immunocytochemistry in single smooth muscle cells. The InsP3R type 1 isofrom (InsP3R1) is highly expressed in colonic smooth muscle, whereas InsP3R type 2 (InsP3R2) and InsP3R type 3 (InsP3R3) are less abundant (Dr S. Currie, personal communication). Confocal images of labelled smooth muscle cells show InsP3R1 were extensively distributed near to the cell surface (Fig. 2Aa). Each punctate region is presumably a cluster of InsP3R. The distribution appeared approximately uniform along the cell and there did not appear to be any region of the cell with a greater expression.

The mAChR type2 (mAChR2) and type 3 (mAChR3) isofroms are present on the plasma membrane of colonic smooth muscle (Sawyer and Ehler, 1998; Zhang et al., 1991). Although the mAChR2 outnumber mAChR3 3:1, the contractile response arises predominantly from mAChR3 activation of PLC-β to evoke InsP3-mediated Ca2+ release (Zhang and Buxton, 1991; Zhang et al., 1991). Selective localisation of mAChR3 in one or few regions of the plasma membrane could cause a greater local agonist-evoked InsP3 production to determine the site of Ca2+ wave initiation. mAChR expression was punctate but mAChR were expressed at various locations along the cell. Any of these sites could be putative Ca2+ wave initiation sites (Fig. 2Ba), i.e. a particular expression of mAChR does not appear to explain the wave initiation site. In control experiments, pre-absorption of either the InsP3R1 or mAChR3 antibody with the immunogen (2:1) prevented binding as shown by an absence of fluorescence (Fig. 2Ab,Bb). Non-specific fluorescence was not detected in experiments using only secondary antibody (not shown). Western blots probed with the anti-InsP3R1 primary antibody show a single clear band (Fig. 2C). Although the InsP3R1 monomer has a molecular weight of 313 kDa, previous reports (Diaz-Muñoz et al., 2008; Fissore et al., 1999) with western blots show the protein running with an apparent molecular weight of 230–260 kDa in accord with the mass observed. In a western blot probed with anti-mAChR3 primary antibody, a clear band occurs near the expected molecular weight for mAChR3 (66 kDa) (Fig. 2D). The remaining bands may be proteolytic degradation products.

From these results it appears that gross structural differences in expression of InsP3R1 and mAChR3 do not determine the site of Ca2+ wave initiation.

**Does the InsP3R cluster size or sensitivity determine the site of Ca2+ wave initiation?**

Although the expression pattern of InsP3R1 does not immediately suggest that differences in distribution of the receptor may determine the site of Ca2+ wave initiation there may be regional differences in Ca2+ sensitivity of InsP3R to InsP3 itself or in InsP3R cluster size to account for the initiation site (Shuai et al., 2006; Smith and Parker, 2009; Swillens et al., 1999). To examine this possibility the local Ca2+ response to a fixed [InsP3] was measured at different locations throughout the cell and compared to the site of initiation of a carbachol-evoked Ca2+ wave. In the representative cell (Fig. 3), carbachol (100 μM) evoked a Ca2+ wave which initiated at a single location (region iii) and propagated the length of the cell (Fig. 3Aa,b). The Ca2+ response was not uniform in all regions of the cell and varied from 1.13 F/F0 at the site of initiation to 2.67 F/F0 (region i) near the nucleus. Next, the location-dependent differences in the sensitivity to InsP3 itself was measured in the same cell. A fixed concentration of caged InsP3 was photolyzed at one minute intervals in 20 μm regions (Fig. 3Ba,b, arrow). The magnitude of InsP3-mediated Ca2+ release at each site (regions i–vi) was compared to the Ca2+ release during a carbachol-evoked Ca2+ wave. In this cell (Fig. 3), the greatest InsP3-evoked Ca2+ release occurred at region i, some 50 μm from the site of wave initiation (region iii). The amplitude of InsP3-mediated Ca2+ release was 1.02 F/F0 (region iii) at the site where the carbachol-evoked Ca2+ wave initiated and 2.78 F/F0 at region i. Similar results were obtained with four other cells (Fig. 3C). With one exception, in each case the site where InsP3-evoked the greatest Ca2+ response was not the location of initiation of the carbachol-evoked Ca2+ wave. These results indicate that the site specificity of agonist-evoked Ca2+ wave initiation is neither due to a greater sensitivity nor increased number of active InsP3R at that location.

Further support for this proposal is found in a separate series of experiments in which subthreshold [InsP3] was applied globally throughout the cell to determine the site of greatest sensitivity and to compare that site with the site of wave initiation. To do this a larger diameter (1.25 mm core-cladding diameter in place of 0.2 mm) fibre optic cable was used for coupling of the xenon flashlamp used for photolysis to the microscope. With a ×40 objective, the area of illumination was ~115 μm in diameter (as determined by coupling the fibre optic cable to a fibre optic illuminator and capturing a brightfield image). The charging voltage applied to the photolysis system’s capacitors, which controls the intensity of the output light pulse, was increased in steps from a sub-threshold level where no Ca2+ rise occurred to the voltage where a clear, but submaximal, Ca2+ response was
first detected (‘Low InsP$_3$’, in the region of 50–90 V). The voltage was then further increased to a level where the maximal Ca$^{2+}$ response was obtained (‘High InsP$_3$’, typically around 200 V). The results show (Fig. 4) that, while the response varies somewhat throughout the cell to subthreshold [InsP$_3$], the most sensitive region was not the wave initiation site (Fig. 4). The data was examined several ways. First, five regions of interest were defined along the cell within illumination zone being spaced at 18 m intervals. The temporal series of mean intensity values for each region were normalised to the local baseline for each event to produce F/F$_0$ values (Fig. 4A). For each event (CCh or flash photolysis), the time from the first rise in Ca$^{2+}$ observed (for whichever region was the first to respond) to the point where the [Ca$^{2+}$], reached half its peak F/F$_0$ value (t$_{1/2peak}$) was measured for each region and plotted versus position (Fig. 4C). The rate of rise provides a measure of sensitivity to InsP$_3$ and was not greatest at the site of wave initiation (5 out of 5 cells).

To help visualise the localised [Ca$^{2+}$] changes in response to CCh and InsP$_3$ a sequential subtraction process was also performed on the entire image stack (Fig. 4D). In this case, pixel by pixel intensity values for each frame were subtracted from the values of the image two frames ahead. This subtraction enabled clear visualisation of areas where there first changes in [Ca$^{2+}$] occurred to subthreshold InsP$_3$ (Fig. 4, dii arrows), which could be compared with the site of wave initiation (Fig. 4di, arrow). In 5 out of 5 cells [Ca$^{2+}$] rose simultaneously...
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at several sites throughout the cell. Again the results suggest that the most sensitive sites are not the region of wave initiation ($n=5$).

**Sites of greater local [InsP$_3$] determined by InsP$_3$R and mAChR colocalisation**

While sensitivity to InsP$_3$ does not offer an explanation for the site of wave initiation it is possible that there are regions of the cell where there are greater [InsP$_3$] increases to activate InsP$_3$R. In these regions InsP$_3$R presumably would open sooner and create faster local Ca$^{2+}$ rises. Sites of faster local rises in Ca$^{2+}$ may occur in regions of increased proximity of mAChR3 and InsP$_3$R1. The proximity of InsP$_3$R1 and mAChR3 to each other was examined by immunocytochemistry and quantified using ImageJ analysis software and JACoP (Just Another Colocalisation Plugin) (Bolte and Cordelieres, 2006). Three-dimensional objects were created from the confocal image z stacks and the centre of mass of each object determined (Fig. 5Aa,Ab,Ba,Bb). Object-based colocalisation and the centre of mass method enables quantification where there are differences in protein expression, as was the case for InsP$_3$R1 and mAChR3. InsP$_3$R1 and mAChR3 colocalisation was quantified by determining the number of centres from one image that overlapped with objects from the other image (Fig. 5Bc). In the cell in Fig. 5, 15.8% of objects throughout the stack colocalised (18 out of 114 objects detected) for mAChR3 and 8.9% of objects (17 out of 191 objects detected) for InsP$_3$R1 (Fig. 5). The images shown are from plane 33 of a 59-plane z-stack, each image was acquired at 150-nm intervals in the axial direction. Similar results were obtained with five other cells; the average colocalisation for mAChR3 was 13.2±2.9% and for InsP$_3$R1 was 9.0±2.3% ($n=6$). These experiments suggest that despite widespread distribution of InsP$_3$R and mAChR there are only a few sites where colocalisation of the receptors occurs. At these sites InsP$_3$R

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Fig. 3. The site of initiation of a carbachol-evoked [Ca$^{2+}$]$_c$ wave does not correlate with the region of greatest InsP$_3$ sensitivity. (A,B) Carbachol (CCh, Ac) evoked an increase in [Ca$^{2+}$]$_c$ (Aa,b) that began at a single site (arrow, Aa, panel 2; region iii shown in panel 1) and propagated from there in either direction (arrows, Aa, panel 3). The [Ca$^{2+}$]$_c$ rises from the regions shown in Aa1 are plotted in Ab. Localised photolysis of caged InsP$_3$ (Bb; arrow) at different sites (regions i to vi; Aa1) along the same cell increased [Ca$^{2+}$]$_c$ locally. [Ca$^{2+}$]$_c$ then passively diffused from the site of release. Interestingly, the InsP$_3$-evoked [Ca$^{2+}$]$_c$ increase (Bb; region iii) at the site where a carbachol-evoked wave initiated (Ab; region iii, green line) was modest when compared with the amplitude of that measured at other sites. The greatest InsP$_3$-evoked [Ca$^{2+}$]$_c$ increase occurred at region i (black line) some 50 μm from the site of wave initiation. The [Ca$^{2+}$]$_c$ images (Aa,Ba) are derived from the time points indicated by the corresponding numbers in (Ab,Bb). [Ca$^{2+}$]$_c$ changes in Aa and Ba are represented by colour; blue is low and red is high [Ca$^{2+}$]. Changes in the fluorescence ratio with time (Ab,Bb) are derived from 3×3 pixel boxes in the second panel in Aa; regions i to vi are drawn as larger yellow circles to facilitate visualisation. A brightfield image of the cell is shown (Aa; left panel); the whole-cell electrode can be seen (left side). The carbachol-containing puffer pipette is located to the right of the cell outside the field of view. (C) Summarised data from five different cells. The Ca$^{2+}$ response at site of greatest InsP$_3$-evoked Ca$^{2+}$ release and that at the wave initiation site of individual cells are shown using connected lines.
may be exposed to faster [InsP₃] increases as compared to other sites in the cell to explain the site of Ca²⁺ wave initiation.

To further explore whether there was a greater extent of InsP₃ mAChR3 colocalisation where the Ca²⁺ wave initiated, this site was compared to another separate site in the same cell. First, the carbachol-evoked InsP₃-mediated Ca²⁺ wave initiation site was established (Fig. 6A). Once this site was determined the cells were fixed and prepared as before for immunocytochemistry. In these experiments to identify each cell a coverslip with an etched grid was used. InsP₃R1 and mAChR3 colocalisation was quantified, in the same cell, at the site of Ca²⁺ wave initiation and at another distant region (Fig. 6B). In these experiments 6±2% of objects colocalised for InsP₃R1 with mAChR3 (n=3) at the site of Ca²⁺ wave initiation. At the region distant from the site of wave initiation 3±2% of objects for InsP₃R1 colocalised with

Fig. 4. CCh evoked a Ca²⁺ wave, and global uncaging of InsP₃ caused [Ca²⁺]c to rise simultaneously throughout the cell. (A) Carbachol evoked a Ca²⁺ wave that initiated from a single site (region 2) and propagated from there as a wave. Low and high levels of InsP₃ uncaging, by varying the voltage used for photolysis (80 V, 90 V and 200 V; see Materials and Methods), resulted in [Ca²⁺]c increasing almost simultaneously throughout the cell. Regions i–v correspond to those in brightfield image (B). (C) Whereas the amplitude of the transient increased with voltage, the time of activation in each region was approximately similar for InsP₃ as revealed by the rise times (t½ to peak). On the other hand, for CCh, the t½ to peak increased with distance from the initiation site. (D) Example frames showing the Ca²⁺ increase ('Initial') during the CCh evoked wave (i) and submaximal (90 V; ii) and maximal (200 V; iii) InsP₃. A sequential subtraction was also performed on the data stacks ('Subtract'), where the pixel intensity values for each frame were subtracted from the values of the image two frames ahead to enable clear visualisation of the regions where the first change in [Ca²⁺]c occurred. The CCh-evoked wave (i) initiation site is shown with an arrow on the subtracted data set. With subthreshold voltage (90 V; ii), InsP₃ release began at three separate areas (arrows). As it is a significantly longer event, the images in the carbachol wave sequence (i) are each three frames apart, whereas the images for the InsP₃ events (ii, iii) are a single frame apart. We found similar results in four additional cells.
mACHR3 (n=3). Thus, there was substantially increased overlap of InsP3R and mACHR3 at the site of Ca\textsuperscript{2+} wave initiation.

**The site of Ca\textsuperscript{2+} wave initiation is dependent on a rapid local Ca\textsuperscript{2+} increase**

The experiments thus far raise the possibility that the site for wave initiation arises from overlap of mACHR3 and InsP3R to generate regions with a more rapid [InsP3] increase. In a first series of experiments to test this possibility [InsP3] was artificially, rapidly increased at a site which was distinct from the usual site of wave initiation. To do this, caged InsP3 was locally photolyzed, releasing a subthreshold [InsP3] during carbachol application. In the first part of the experiment carbachol (100 μM; Fig. 7Ci) was applied to verify the Ca\textsuperscript{2+} wave initiation site (region ii in Fig. 7Ai frame 1, yellow arrow in Fig. 7Ai frame 2); the wave then propagated the length of the cell (Fig. 7Ai,Bi). Caged InsP3 was next photoreleased locally at a site distant (50 μm) from that of wave initiation (20 μm diameter; region iv in Fig. 7Ai), using an empirically determined amount which did not trigger any SR Ca\textsuperscript{2+} release (Fig. 7Bii). Finally, carbachol was again applied (~90 seconds later) but this time subthreshold InsP3 was photolysed before the Ca\textsuperscript{2+} wave could initiate. Upon photorelease of caged InsP3 a Ca\textsuperscript{2+} wave initiated (yellow arrow in Fig. 7Aii frame 8) at the site of photolysis (region iv shown in Fig. 7Ai) and propagated from there (Fig. 7Aii,Bii). These results support the proposal that the site of wave initiation occurs in regions of the greatest [InsP3] increase in which the photolyzed InsP3 adds to that produced by carbachol, thereby raising InsP3 concentration over the threshold for wave initiation to occur (n=4).

In the next series of experiments to determine the contribution of the local [Ca\textsuperscript{2+}]<sub>c</sub> increase to the specificity of the initiation site, the Ca\textsuperscript{2+} rise was selectively attenuated only at the initiation site by local photorelease of the caged Ca\textsuperscript{2+} buffer diazo-2.

First, control experiments were carried out to confirm the effectiveness of diazo-2 in buffering [Ca\textsuperscript{2+}], in small, restricted locations of the cell during membrane depolarisation and voltage-dependent Ca\textsuperscript{2+} entry (I<sub>Ca</sub>) (Fig. 8). A voltage-clamped colonic myocyte depolarised from -70 mV to +10 mV activated I<sub>Ca</sub> and produced transient, uniform, reproducible elevations in [Ca\textsuperscript{2+}] throughout the cell (Fig. 8Aa,b). One minute later the cell was again depolarised but this time the caged Ca\textsuperscript{2+} chelator diazo-2 (100 μM) was photoreleased to buffer the Ca\textsuperscript{2+} in one small (20 μm) region of the cell 100 ms prior to the depolarisation. Photorelease of diazo-2 selectively attenuated the Ca\textsuperscript{2+} rise at the site of photolysis whereas the [Ca\textsuperscript{2+}]<sub>c</sub> increases at other locations were unaffected (Fig. 8Aa,e). In another experiment on this same cell diazo-2 was photoreleased 800 ms after membrane depolarisation and it rapidly buffered the [Ca\textsuperscript{2+}]<sub>c</sub> increase at that location as shown by an instantaneous decrease in [Ca\textsuperscript{2+}]<sub>c</sub> (Fig. 8Ba,e). Again, other locations throughout the cell were unaffected. These experiments confirm diazo-2 rapidly and effectively buffers local [Ca\textsuperscript{2+}]<sub>c</sub> increases after photolysis.

Diazo-2 was next used to determine whether or not the site of Ca\textsuperscript{2+} wave initiation required regions of faster increases in [Ca\textsuperscript{2+}]<sub>c</sub>. The InsP3-generating agonist carbachol (100 μM; Fig. 9Ci) produced a Ca\textsuperscript{2+} wave that initiated at a single location and propagated from that site along the length of the cell (Fig. 9Ai,Bi). There was a latency of 5.3 sec from the start of agonist stimulation to Ca\textsuperscript{2+} wave initiation (Fig. 9Ai,Bi). In the
next part of the experiment (~90 sec later) caged Ca^{2+} buffer diazo-2 was photoreleased (Fig. 9Dii) at the wave initiation site after carbachol application (Fig. 9Cii) but before the Ca^{2+} wave initiated. Photolysis of diazo-2 prevented the increase in [Ca^{2+}]_c at the wave initiation site and interestingly the carbachol-evoked Ca^{2+} wave moved from the primary location after a latency of 6.6 sec, to initiate at a secondary location (Fig. 9Aii, Bii). In five other cells diazo-2 effectively prevented the Ca^{2+} wave from initiating at the primary ‘eager’ location and after a delay generated a secondary location which produced the Ca^{2+} wave. The average delay from the start of carbachol application to Ca^{2+} wave initiation under control conditions was 4.23±2.5 ms for waves occurring at the first site and 5.40±2.6 ms at the second site after diazo-2 photolysis (*P<0.05*) (Fig. 9E). These experiments suggest that a characteristic of the initiation site is a region of most rapid Ca^{2+} rise. It appears that if the primary ‘eager’ site of Ca^{2+} wave initiation is compromised the cell maintains the capability to initiate a Ca^{2+} wave at a new ‘eager’ location. Together, these results suggest that the site of Ca^{2+} wave initiation arises from an increased rate of rise of Ca^{2+} at the ‘eager’ location presumably due to faster local [InsP_3] increase as a result of colocalisation of InsP_3R and mAChR.

**Discussion**

In several cell types a characteristic response to InsP_3-generating agonists is a Ca^{2+} rise that initiates at a small single site then propagates along the length of the cell as a Ca^{2+} wave (Bootman et al., 1997a; Marchant and Parker, 2001; McCarron et al., 2010; Straub et al., 2000; Thorn et al., 1993). Interestingly, the site at which Ca^{2+} waves initiate is often fixed and constant during each
agonist activation. The present study examined the mechanisms that determine the site of agonist-evoked Ca\textsuperscript{2+} wave initiation in smooth muscle and the results suggest waves initiate where \([\text{InsP}_3]\) and \([\text{Ca}^{2+}]_c\) increases most rapidly as a result of colocalisation of \(\text{InsP}_3\)R1 and mAChR3. 

InsP\textsubscript{3}R were found to be distributed widely throughout the cell in the present study but with increased occurrence at the nucleus and plasma membrane as was also seen in intestinal myocytes (and Zholos, 2004). The \(\text{InsP}_3\)-generating muscarinic receptor, mAChR3, was also approximately uniformly distributed in the plasma membrane. The distribution of each receptor does not explain the site of wave initiation. However, when mAChR3 and \(\text{InsP}_3\)R1 were dual labelled and the distribution quantified by centre of mass analysis, there was a small number of sites which showed co-existence of the two receptors. A 13% overlap between mAChR3 and \(\text{InsP}_3\)R1 and 9% overlap of \(\text{InsP}_3\)R1 and mAChR3 existed. The much higher expression of \(\text{InsP}_3\)R1 than mAChR3 accounts for the difference in percent overlap. This observation raised the possibility that regions of overlap may explain the site of origin of the Ca\textsuperscript{2+} wave. To examine this possibility a Ca\textsuperscript{2+} wave was first evoked then immunocytochemistry carried out in the same cell to determine the extent of overlap of \(\text{InsP}_3\)R1 and mAChR3 at the wave initiation site. A significantly greater overlap of the receptors existed at the site of wave initiation than in other regions of the cell. The colocalisation of mAChR3 and \(\text{InsP}_3\)R1 may create regions where \(\text{InsP}_3\) has little distance to diffuse to activate \(\text{InsP}_3\)R to generate faster and greater Ca\textsuperscript{2+} release at these sites. This arrangement will produce an effective \(\text{InsP}_3\) junction in which mAChR-generated \(\text{InsP}_3\) is preferentially and rapidly delivered to particular \(\text{InsP}_3\)R. \(\text{InsP}_3\) acts in these circumstances as a highly localised and targeted signal rather than a diffusible second messenger. Indeed in SH-SY5Y cells the majority of sites of local Ca\textsuperscript{2+} release (Ca\textsuperscript{2+} puffs) were within 100 nm of the plasma membrane (Smith et al., 2009). In addition, a Ca\textsuperscript{2+}-activated K\textsuperscript{+} current was activated by \(\text{InsP}_3\)-evoked Ca\textsuperscript{2+} release (Hoesch et al., 2004). These results suggest a prevalence of active \(\text{InsP}_3\)R near the plasma membrane.

A prediction from the proposal that waves initiate where \(\text{InsP}_3\)R and mAChR co-localised is that at these sites \([\text{InsP}_3]\) and
may increase most rapidly. To test this possibility two experiments were undertaken. In the first, localised increases in InsP3 at sites distant from the normal initiation site were evoked more rapidly than the InsP3 increases that occur by the mAChR3 agonist carbachol to short circuit the wave initiation process. InsP3 was rapidly and locally increased by photolysis of a subthreshold amount of caged InsP3 during application of the InsP3-generating agonist (carbachol). The local photolysis resulted in the Ca2+ wave initiation site shifting to the region of InsP3 release and the wave travelling along a new path.

In the second experiment, the Ca2+ rise at the site of wave initiation, and only this site, was attenuated using targeted localised photolysis of the caged Ca2+ buffer diazo-2. When the Ca2+ was rapidly buffered during carbachol application this again resulted in the Ca2+ wave shifting from the normal site and initiating at a new secondary site after a short delay. There are only a few regions of the cell where mAChR and InsP3 colocalised and if the Ca2+ increase at the normal site of Ca2+ wave initiation is compromised then presumably one of these other sites will become the wave initiation site. Together, these results suggest InsP3 derived from activation of particular mAChR3 is selectively delivered to InsP3R via the close apposition of the two receptors to generate the Ca2+ wave initiation site.

Directed, selective communication between processes in cells drives intracellular signalling. Sites of privileged communication also coordinates activities among InsP3R and various proteins involved in intracellular Ca2+ signalling to generate selective responses (Delmas et al., 2002; Hur et al., 2005; Tovey et al., 2008; Yuan et al., 2005). For example, some agonists acting via G protein coupled receptors to release Ca2+ via InsP3R evoke specific responses by being additionally coupled to adenylate cyclase (AC) to create a local signalling complex. Parathyroid hormone-stimulated cyclic adenosine monophosphate (cAMP) formation was delivered locally from AC (AC6) to InsP3R (Tovey et al., 2008). Changes in the fluorescence ratio with time (Ab,Be,Bc) are derived from 3×3 pixel boxes shown in the far right panel in Aa; regions i to iii are drawn as larger yellow circles to facilitate visualisation. A brightfield image of the cell (Aa; right panel); the whole-cell electrode can be seen (left side) and photolysis spot is indicated.
InsP$_3$R1 phosphorylation and sensitisation and Ca$^{2+}$ release (Hur et al., 2005). Together these studies highlight the significance of local coupling events to global cell signalling procedures.

Signalling complexes may also explain the site of Ca$^{2+}$ wave initiation. Localised expression of particular proteins sensitise the response to InsP$_3$ and correlate with the wave initiation site in some cells. In cultured rat hippocampal neurons and pheochromocytoma cells the wave initiation site was associated with localised expression of the ER luminal protein chromogranin B, a protein which sensitises InsP$_3$ with localised expression of the ER luminal protein chromogranin B, a protein which sensitises InsP$_3$ to InsP$_3$ and phosphatidylinositol-4-phosphate kinase (PIPKI$\gamma$), a kinase involved in the production of the InsP$_3$ precursor phosphatidyl inositol 4,5-bisphosphate (PIP$_2$) (Jacob et al., 2005). In HeLa cells local Ca$^{2+}$ release usually initiated from a single perinuclear Ca$^{2+}$ puff site although InsP$_3$R were expressed evenly around the nucleus (Thomas et al., 2000). The perinuclear sites produced multiple Ca$^{2+}$ puffs during prolonged histamine stimulation and often became the site where a Ca$^{2+}$ wave initiated (Bootman et al., 1997b). The location and frequency of Ca$^{2+}$ puffs were dependent on [InsP$_3$], where some sites appeared to have a greater sensitivity to InsP$_3$ and Ca$^{2+}$ release occurred sooner (Bootman et al., 1997b; Marchant and Parker, 2001).

Regional differences in sensitivity to InsP$_3$ do not appear to determine the location of Ca$^{2+}$ wave initiation in the present study. We examined this possibility by comparing the amplitude of [Ca$^{2+}$]$_c$ increase during local photolysis of caged InsP$_3$, to the magnitude of Ca$^{2+}$ release during carbachol-evoked Ca$^{2+}$ waves at several sites in the cell including the initiation site. The magnitude of Ca$^{2+}$ release during a fixed [InsP$_3$] was not greatest at the site of wave initiation as would have been expected if the sensitivity to InsP$_3$ was greatest here. Indeed, InsP$_3$-evoked Ca$^{2+}$ increase at the wave initiation site was significantly smaller than for other regions of the cell. These experiments suggest that differences in the sensitivity to InsP$_3$ do not determine the site of Ca$^{2+}$ wave initiation in smooth muscle. Consistent with our results, others found no difference in the frequency and sensitivity of perinuclear and cytosolic Ca$^{2+}$ puffs to the [InsP$_3$] stimulation threshold (Smith et al., 2009).

The differences in the above results and in the apparent sensitivities to InsP$_3$ could be explained by different methodological approaches. Thomas and colleagues (Thomas et al., 2000) used agonists to evoke Ca$^{2+}$ puffs whereas Smith and colleagues (Smith et al., 2009) used a membrane permeant photolabile InsP$_3$ analogue. In the first instance responses to InsP$_3$
produced in microdomains would be measured and in the second
photolysis would raise the [InsP₃] even throughout the cell. The
differences in Ca²⁺ puff characteristics measured at different puff
sites could be explained by microdomains of synthesis and diffusion of InsP₃ as proposed in the present study.

Nerve released acetylcholine is directed to specific regions of the smooth muscle cell via the close apposition (20 nm) of muscle and nerve at the synapse (Fausone-Pellegrini et al., 1989; Goyal and Chaudhury, 2010; Mitsui and Komuro, 2002; Silva et al., 1968) and presumably would create microdomains of higher concentration of the agonist the there. The site of Ca²⁺ wave initiation in smooth muscle arises from a complex where mAChR3 and InsP₃R1 are structurally and functionally coupled to generate junctions in which InsP₃ acts as a highly localised signal by being rapidly and selectively delivered to particular InsP₃R.

Materials and Methods
Cell isolation
Male guinea-pigs (350–500 g) were humanely killed by cervical dislocation
followed by immediate exsanguination in accordance with the guidelines of the Animal (Scientific Procedures) Act UK, 1986. A segment of intact distal colon (∼5 cm) was transferred to oxygenated (95% O₂, 5% CO₂) physiological saline solution comprising (mM): 118.4 NaCl, 25 NaHCO₃, 4.7 KCl, 1.13 NaH₂PO₄, 1.3 MgCl₂, 2.7 CaCl₂ and 11 glucose (pH 7.4). Following removal of the mucosa and longitudinal muscle layer from the tissue, single smooth muscle cells, largely from circular muscle, were enzymatically dissociated (McCarron and Murr, 1999). All experiments were carried out at room temperature (20–22°C) unless otherwise noted.

Electrophysiology
Cells were voltage-clamped using conventional tight-seal whole-cell recording methods. The extracellular solution contained (mM): 80 Na glutamate, 40 NaCl, 20 tetraethylammonium chloride (TEA), 1.1 MgCl₂, 3 CaCl₂, 10 HEPES and 30 glucose (pH 7.4 with NaOH). The pipette solution contained (mM): 85 Cs₂SO₄, 20 CsCl, 1 MgCl₂, 30 HEPES, 3 MgATP, 2.5 pyruvic acid, 2.5 malic acid, 1 NaH₂PO₄, 5 creatine phosphate, 0.025 caged inositol, 1.4,5-trisphosphate (InsP₃) triosodium salt. Whole-cell currents were measured using an Axopatch 200B (Axon Instruments, Union City, CA), low-pass filtered at 500 Hz (8-pole Bessel filter; Frequency Devices, Haverhill, MA), digitally sampled at 1.5 kHz using a Digidata interface and pClamp (version 8, Axon Instruments) and stored for analysis.

Ca²⁺ imaging
Single, freshly isolated colonic smooth muscle cells were loaded with the Ca²⁺-sensitive dye fluo 3 acetoxymethyl ester (AM) (10 µM; to prevent contraction) for at least 20 min before the start of the experiment. The 20–30 min was sufficient to allow intracellular esterases to hydrolyze the AM moiety. Two-dimensional [Ca²⁺], images were obtained using a wide-field digital imaging system (Olson et al., 2010). Single cells were illuminated at 488 nm (bandpass 14 nm) from a monochromer (Polychrome IV, T.I.L.L. Photonics, Martinsried, Germany) and imaged through an oil-immersion objective lens. The diameter of the illumination zone and whose carbachol initiation site also fell within the zone were used. Although a >20 objective increased the photolysis illumination area, such that the majority of cells could generally be positioned entirely within the illumination zone, the image resolution obtained with this lens was inadequate for detailed image analysis.

The charging voltage applied to the photolysis system’s capacitors, which controls the intensity of the output light pulse, was increased in steps from a sub-threshold level where no Ca²⁺ rise occurred to the voltage where a clear, sub-millisecond Ca²⁺ response was first detected (Low InsP₃, in the region of 50–90 V). The voltage was then further increased to a level where the maximal Ca²⁺ response was obtained ('High InsP₃', typically around 200 V).

Immunocytochemistry
Smooth muscle cells were placed on to microscope slides and allowed to settle for 60 min in a humidified environment. Cells were fixed (15 min) in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8 mM NaH₂PO₄, 0.7M, pH 7.4), permeabilized with 0.1% Triton X-100 in PBS (10 min), then incubated with primary antibodies (2 hours) in an antibody buffer (150 mM NaCl, 15 mM Na₂Citrate, 2% goat serum, 1% BSA, 0.05% Triton-X100). Incubation with secondary antibodies was for 1 h. InsP₃R1 was detected using monoclonal InsP₃R1 antibodies (1:50), mACHR was detected using an isofrom-specific polyclonal antibody (1:40). Primary antibodies were visualised by fluorescence confocal microscopy using fluorescently conjugated secondary antibodies.

The cells were imaged using a Leica SP5 upright confocal microscope (Leica Microsystems UK, Milton Keynes, UK). The excitation beam was produced by an argon (488 nm) or HeNe (543 and 643 nm) laser and delivered to the sample via an oil immersion objective lens (LCX, PL APO×63 1.40 NA). Emitted fluorescence was captured using acousto-optical tuneable filters coupled to a PMT controlled by Leica imaging software. Full frame images (246 µm×246 µm) with a pixel size of 240.5 µm at the cell were acquired. Z stacks were acquired as 3 x-y image averages at 150-nm intervals. The scanner angle was further adjusted to obtain images at 6X magnification and a pixel size of 40x40 µm.

Western blotting
Tissue was frozen in liquid N₂ immediately following dissection and homogenised (200 mg tissue per ml) in either RIPA buffer or in a Triton-based lysis buffer (20 mM Tris Base, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA (pH 7.4), both containing protease inhibitors, by grinding with eppendorf-tube pestles and repeatedly vortexing. Homogenates were clarified by centrifugation (5 min at 13,200 rpm) and the supernatant aliquoted into 100 µl volumes and stored at −80°C. Prior to running a western blot, the lysate was diluted 4-fold in lysis buffer. For each lane to be run, a sample was prepared by adding 5 µl 5x-PLB (40 mM glycerol (18%), 120 mM 10% SDS (5.5%), 50 mM 1 M Tris (pH 6.5) (0.23 M), 50 mg Bromeophenol Blue (0.023%), 10 µl [l-mercaptoethanol (4.5%)] to an 1:10000 dilution of lsd (i.e. each lane contained lsd from −1 mg tissue). The samples were then kept at 99°C for 5 min and pulse centrifuged then loaded into polyacrylamide gels (7% for InsP₃R blot, 10% for mACHR), along with 10 µl H₂O. Prestained HMW Protein Standard, and run at 200 V for 1–2 h (2 h for 7% gel, 1 h for 10% gel) using a 0.25 M Glycine, 0.025 M Tris, 0.1% SDS running buffer. The proteins were then transferred to a nitrocellulose membrane by electrophoretich for 1.5–2.5 h (1.5 h for mACHR blot, 2 h for InsP3R blot), using a Bio-Rad Mini Trans-Blot cell with a limiting voltage/current of 100 V/400 mA and a 25 mA Tris, 192 mM Glycine, 20% MeOH, pH 8.0 transfer buffer. After electrophoretich, the membrane was removed and blocked in a 5% nonfat milk powder solution in PBS overnight at 4°C. After two 5 min washes in PBS-T (0.05% Tween in PBS), the membrane was probed with a 1:500 primary antibody dilution (rabbit polyclonal anti-mACHR, and mouse monoclonal anti-InsP₃R1) in 5% milk powder in PBS-T overnight. After three 5 min and two 15 min washes, the membrane was incubated in a 1:1000 secondary antibody dilution (anti-mouse-HRP and anti-rabbit-HRP) in 5% milk powder in PBS-T for 1 h followed by two 5 min and two 10 min washes. All washes and antibody incubations were carried out at room temperature on an orbital shaker (−50 rpm). The protein bands were then detected by enhanced chemiluminescence.

A xenon flashlamp (Rapp Optoelectronic, Hamburg, Germany) was used to photolyze caged InsP₃ or the caged Ca²⁺ buffer diazo-2. The flashlamp output was passed through a UV-5 filter to select ultraviolet light, focused and directed to the illumination light path through a fibre-optic bundle andlow-pass dichroic mirror at the lens part of the epi-illumination attachment of the microscope. The diameter of the fibre optic together with the lens magnification determined the area (spot size ~20 µm) of photolysis (McCarron and Olson, 2008). The output intensity of the flash lamp was 0.19 mW at the objective lens. The timing of photolysis was recorded using pClamp software by using a TTL output from the flashlamp.
using Pierce’s ECL Western Blotting Substrate and imaged using an X-OMAT system.

Data analysis

[Ca\textsuperscript{2+}]\textsubscript{i} images were analysed using the program Metamorph 7.1.3 (Molecular Devices, Wokingham, UK). Changes in fluorescence were expressed as ratios (F/F₀ or ΔF/F₀) of fluorescence counts (F) relative to baseline (control) values (taken as 1) before stimulation (F₀). Peak height was subtracted from the average baseline value derived from the 100 frames before carbachol application or flash photolysis of caged InsP\textsubscript{3}. The delay in the onset of the Ca\textsuperscript{2+} wave after application of carbachol was measured as the time from the start of carbachol application for [Ca\textsuperscript{2+}]\textsubscript{i}, to an increase by 10% F/F₀ of the peak amplitude. Colocalisation of InsP\textsubscript{3},R1 and mACHR3 was quantified using Image v1.44 analysis software (Rashband, 1997–2011) and JACoP v2.0 (Just Another Colocalisation Plugin) (Bolte and Cordelières, 2006) to determine object based colocalisation. Briefly, the confocal image stacks were smoothed using a 3×3 median filter and an image mask applied. Three-dimensional objects were created using the 3D object counter plugin from the regions of the images with fluorescence above a threshold value. The centre of mass of each object was then determined. InsP\textsubscript{3},R1 and mACHR3 colocalisation was quantified by determining the number of centres from one image that were colocalised with objects from the other image.

Summarised results are expressed as means ± s.e.m. of n cells. A paired or unpaired Student’s t-test was applied to the raw data, as appropriate, P<0.05 was considered significant.

Global InsP\textsubscript{3} photolysis analysis

The raw fluorescence data was analysed using Metamorph. First the data was smoothed using a 3-frame rolling average applied to the sequence of captured images so that the [Ca\textsuperscript{2+}]\textsubscript{i} signal increased with submaximal InsP\textsubscript{3} could be resolved. Five regions of interest were then defined along the cell within the illumination zone, one of which was at the CCh wave initiation site, with the others being spaced at ≈18 μm intervals. The temporal series of mean intensity values for each region were exported to the software Microcal Origin, in which the data was normalised to the local baseline for each event to produce F/F₀ values. For each event, the time from the first rise in [Ca\textsuperscript{2+}]\textsubscript{i} was observed (for whichever region was the first to respond) to the point where the [Ca\textsuperscript{2+}]\textsubscript{i} had reached half its peak F/F₀ (τ\textsubscript{½}) was measured for each region and plotted versus position.

Using Metamorph, a sequential subtraction process was also performed on the rolling average sequences, where the pixel intensity values for each frame were averaged to provide an image of fluorescence, relative to a rolling average. This enabled the clear visualisation of the region where the first changes in [Ca\textsuperscript{2+}]\textsubscript{i} occurred and, in the case of CCh, of wave progression.

Drugs and chemicals

Concentrations in the text refer to the salts, where appropriate. Fluo-3 AM, diazot-2 tetra-potassium salt, goat anti-rabbit and goat anti-mouse secondary antibodies (Alexa 488, TMRE, Alexa 647) and HiMark Prestained HMW Protein Standard were purchased from Invitrogen (Paisley, UK) and caged InsP\textsubscript{3} (Alexa 488, TMRE, Alexa 647) and HiMark Prestained HMW Protein Standard were purchased from Pierce (Thermo Fisher Scientific, Northumberland, UK). All other reagents were purchased from Sigma (Poole, UK).

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