Agonist-induced phasic and tonic responses in smooth muscle are mediated by InsP₃

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Summary
Many cellular functions are regulated by agonist-induced InsP₃-evoked Ca²⁺ release from the internal store. In non-excitatory cells, predominantly, the initial Ca²⁺ release from the store by InsP₃ is followed by a more sustained elevation in [Ca²⁺]; via store-operated Ca²⁺ channels as a consequence of depletion of the store. Here, in smooth muscle, we report that the initial transient increase in Ca²⁺, from the internal store, is followed by a sustained response also as a consequence of depletion of the store (by InsP₃), but, influx occurs via voltage-dependent Ca²⁺ channels. Contractions were measured in pieces of whole distal colon and membrane currents and [Ca²⁺]; in single colonic myocytes. Carbachol evoked phasic and tonic contractions; only the latter were abolished in Ca²⁺-free solution. The tonic component was blocked by the voltage-dependent Ca²⁺ channel blocker nimodipine but not by the store-operated channel blocker SKF 96365. InsP₃ receptor inhibition, with 2-APB, attenuated both the phasic and tonic components. InsP₃ may regulate tonic contractions via sarcolemma Ca²⁺ entry. In single cells, depolarisation (to ~−20 mV) elevated [Ca²⁺]; and activated spontaneous transient outward currents (STOCs). CCh suppressed STOCs, as did caffeine and InsP₃. InsP₃ receptor blockade by 2-APB or heparin prevented CCh suppression of STOCs; protein kinase inhibition by H-7 or PKC₁₀₉,₃₆ did not. InsP₃ suppressed STOCs by depleting a Ca²⁺ store accessed separately by the ryanodine receptor (RyR). Thus depletion of the store by RyR activators abolished the InsP₃-evoked Ca²⁺ transient. RyR inhibition (by tetracaine) reduced only STOCs but not the InsP₃ transient. InsP₃ contributes to both phasic and tonic contractions. In the former, muscarinic receptor-evoked InsP₃ releases Ca²⁺ from an internal store accessed by both InsP₃ and RyR. Depletion of this store by InsP₃ alone suppresses STOCs, depolarises the sarcolemma and permits entry of Ca²⁺ to generate the tonic component. Therefore, by lowering the internal store Ca²⁺ content, InsP₃ may generate a sustained smooth muscle contraction. These results provide a mechanism to account for phasic and tonic smooth muscle contraction following receptor activation.

Key words: Smooth muscle, Calcium, STOCs

Introduction
Agonist-induced increases in the cytosolic Ca²⁺ concentration ([Ca²⁺];) produce smooth muscle contraction characterised by a fast initial peak, the phasic component, followed by a decline to a lower maintained tension level, the tonic component. Hitherto, the phasic component has been attributed to an InsP₃-evoked release of Ca²⁺ from the sarcoplasmic reticulum (SR) store; the tonic component from Ca²⁺ entry via the sarcolemma following depolarisation, independently of InsP₃ (Baron et al., 1984; Somlyo et al., 1985; Kobayashi et al., 1989). This explanation may now require review; for example, muscarinic receptor-induced depolarisation, which underlies the tonic component, is inhibited in smooth muscle cells that lack InsP₃ receptors (Suzuki et al., 2000). If, as is implied in this result, InsP₃ is implicated in the tonic response, the question arises as to how it can release sufficient Ca²⁺ (from SR stores of finite capacity) to generate both the sustained changes in membrane potential and contraction characteristic of the tonic response, and the Ca²⁺ required for the phasic component.

Ca²⁺ release from the SR store is controlled by two receptor-channel complexes: the ryanodine receptor (RyR), which mediates Ca²⁺-induced Ca²⁺ release (CICR); and the InsP₃ receptor, which is involved in transmitter/ligand activity at the sarcolemma. Stores are classified, on the basis of the receptors they express, into ryanodine-sensitive and InsP₃-sensitive stores. Whether or not a single store or multiple stores containing different arrays of receptors exist in smooth muscle is controversial (e.g. Bolton and Lim, 1989; Yamazawa et al., 1992; Golovina and Blaustein, 1997; Sims et al., 1997; Janiak et al., 2001). We have recently proposed the existence of two stores in smooth muscle, one with exclusively RyR, the other with both RyR and InsP₃ receptors (Flynn et al., 2001) (see also Baró and Eisner, 1995).

SR store receptor activation releases Ca²⁺ to generate intracellular signals, the best known of which are the Ca²⁺ ‘sparks’ – spontaneous transient releases of Ca²⁺ from the RyR (reviewed by Bootman and Lipp 1999; Niggli, 1999; Jaggar et al., 2000; Bootman et al., 2001; Sanders, 2001). Ca²⁺ sparks in turn activate a number of intracellular effectors including large conductance Ca²⁺-activated K⁺ channels (BKCa) (Nelson et al., 1995) (reviewed by Berridge, 1997), which regulate [Ca²⁺], and a variety of smooth muscle contractile responses (e.g. Nelson et al., 1995; Ganitkevich and Isenberg, 1996; Kume et al., 1995; Khan et al., 1998; Porter et al., 1998). The activation of up to 100 BKCa, by a Ca²⁺ spark from RyR, gives rise to spontaneous transient outward currents (STOCs
Ca\(^{2+}\) from the SR Ca\(^{2+}\) store) but also for the tonic component

The relationship between STOCs and muscarinic agonists that possibility was explored in the present study and the generation of Ins\(_3\) was examined using whole cell patch-clamp and fluorescence techniques in single dissociated smooth muscle cells. In particular, the relationship between Ins\(_3\) receptor activity and the ability to suppress STOCs and regulate contraction was examined. The results show that Ins\(_3\) receptor activity can account for the suppression of STOCs by cholinergic agonists and raise the possibility that generation of Ins\(_3\) accounts not only for the phasic component (by releasing Ca\(^{2+}\) from the SR Ca\(^{2+}\) store) but also for the tonic component of contraction since Ins\(_3\), by depleting the SR store, suppresses STOCs and so depolarises the sarcolemma and facilitates entry via voltage-dependent Ca\(^{2+}\) channels.

Materials and Methods

Materials

Fluo-3 penta-ammonium salt was purchased from Molecular Probes (Cambridge Bioscience, Cambridge, UK), caged-Ins\(1,4,5\)P\(_3\)-trisodium salt, ryanodine and SKF-96365 were from Calbiochem-Novabiochem (Nottingham, UK), and nimodipine was from Research Biochemical (Nottingham, UK). The protein kinase C inhibitory peptide, PKC\(_{19-36}\) [PKC IC\(_{50}\) 0.15 \(\mu\)M (House and Kemp, 1987; Malinow et al., 1989)], had the sequence RFARKGALRQKNVHEVKN and was synthesized by Alexis (Nottingham, UK). All other reagents were purchased from Sigma (Poole, UK). Caffeine (10 \(mM\)) and carbachol chloride (CCh; 50 \(mM\) or 100 \(mM\)) were each applied by hydrostatic pressure ejection (PicoPump PV 820, WPI, Aston, UK) and Ins\(_3\) was liberated by flash photolysis of the caged compound (see below). PKC\(_{19-36}\) and heparin were each dissolved in the pipette solution and introduced into the cells via the patch pipette. Other drugs [atropine sulphate, tetraacaine HCl, triethylammonium chloride (TEA)] dissolved in water, unless otherwise stated, were each perfused into the solution bathing the cells (~5 ml per minute). Nimodipine was dissolved in 95% ethanol; final bath concentration of the solvent was 0.05%. 2-APB was dissolved in di-methyl sulfoxide (final bath concentration of the solvent was 0.005%) as were 1-(5-isopropinolinosulfonyl)-2-methylpiperazine (H-7), indolactam, 2-aminoethoxydiphenyl borate (2-APB), SKF 96365 and ryanodine (bath concentration of solvent <0.1%).

Methods

From male guinea-pigs (500-700 g), killed by cervical dislocation then immediately bled following the guidelines of the Animal (Scientific Procedures) Act 1986, a segment of the intact distal colon (~5 cm) was removed and transferred to a Sylgard-coated (Dow Corning) Petri dish containing an oxygenated (95% \(O_2\), 5% \(CO_2\)) physiological saline solution (PSS; 118.4 mM NaCl, 25 mM NaHCO\(_3\), 4.7 mM KCl, 1.13 mM NaH\(_2\)PO\(_4\), 1.3 mM MgCl\(_2\), 2.7 mM CaCl\(_2\) and 11 mM glucose, pH 7.4).

Pieces of intact colon were cleaned, by perfusing with PSS, then mounted in a vertical, heated organ bath (10 ml, 37°C) filled with oxygenated PSS (95% \(O_2\), 5% \(CO_2\)). One end of each piece was fixed to a hook on the bottom of the bath and the other attached to a force displacement transducer (Grass FT03C). Contractions were recorded (Grass polygraph, Model 79E) in response to drugs added to the bath (5-300 \(\mu\)l). Drugs were washed out by emptying and refilling the bath. Ca\(^{2+}\)-free solutions were prepared without compensation. Signals were digitized (10 Hz; Data translation board 2801-A) using a software program (kindly provided by F. L. Burton, University of Glasgow). To compare contractile responses to agonists, before and after 2-APB, the phasic component was taken as the force developed at the time required to reach 80% of the initial (control) peak contraction minus baseline. The tonic response was measured as the force developed from an average of 50 seconds of recording beginning 3.3 minutes (2000 data points) after agonist was added, minus baseline.

Single smooth muscle cells were enzymatically dissociated from guinea-pig colonic muscle (McCarron and Muir, 1999). Membrane currents were measured using conventional tight seal whole-cell recording. The composition of the extracellular solution was: 80 mM Na glutamate, 60 mM NaCl, 4.7 mM KCl, 1.1 mM MgCl\(_2\), 3 mM CaCl\(_2\), 10 mM Heps and 10 mM glucose, pH 7.4 with 1 M NaOH. Tetraethylammonium chloride (20 \(mM\)), where used, replaced equimolar amounts of NaCl. Ca\(^{2+}\)-free solutions contained MgCl\(_2\) (3 \(mM\)) and ethylene glycol-bis(\(\beta\)-aminoethly ether)N,N,N,N’-tetra-acetic acid (EGTA, 1 \(mM\)). Unless otherwise stated, the pipette solution contained: 105 mM KCl, 1 mM MgCl\(_2\), 3 mM MgATP, 2.5 mM Pyruvic acid, 2.5 mM Malic acid, 1 mM NaH\(_2\)PO\(_4\), 5 mM creatine phosphate, 0.5 mM guanosine triphosphate, 30 mM Heps, 0.1 mM fluo-3 penta-ammonium salt and 25 \(\mu\)M caged Ins\(1,4,5\)P\(_3\) trisodium salt, pH 7.2 with 1 M KOH. Whole-cell currents were amplified by an Axopatch 1D (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 Axotape (Axon Instruments) and stored for analysis.

[Ca\(^{2+}\)] was measured using the membrane-impermeable dye fluo-3 (penta-ammonium salt) introduced into the cell from the patch pipette. Fluorescence was measured using a microfluorimeter that comprised an inverted fluorescence microscope (Nikon diaphot) and a photomultiplier tube with a bi-alkali photocathode. Fluo-3 was excited at 488 nm (bandpass 9 nm) from a PFI Delta Scan (Photon Technology International, East Sheen, London, UK) through the epi-illumination port of the microscope (using one arm of a bifurcated quartz fiber optic bundle). Excitation light was passed through a field stop diaphragm to reduce background fluorescence and reflected off a 505 nm long-pass dichroic mirror; emitted light was guided through a 535 nm barrier filter (bandpass 35 nm) to a photomultiplier in photon counting mode. Longer wavelengths, from bright field illumination with a 610 nm Shott glass filter, were reflected onto a CCD camera (Sony model XC-75) mounted on to the viewing port of the Delta Scan allowing the cell to be monitored during experiments. Interference filters and dichroic mirrors were obtained from Glen Spectra (London, UK). To photolyse caged-Ins\(1,4,5\)P\(_3\) (referred to in the text as Ins\(_3\)) the output of a xenon flashlamp (Rapp Optoelektronic, Hamburg, Germany) was passed through a UG-5 filter to select ultraviolet light and merged into the excitation light path of the microfluorimeter using the second arm of the quartz bifurcated fiber optic bundle. The nominal flash lamp energy was 57 \(mJ\) measured at the output of the fiber optic bundle and the flash duration was about 1 millisecond. Single cell experiments were conducted at room temperature (18-22°C).
STOCs were activated by slowly depolarising the membrane potential from \(-70\) mV to avoid activation of a large Ca\(^{2+}\) current. STOCs varied widely in amplitude, duration and frequency; therefore, to summarize STOC activity, they were integrated for 5 or 20 second periods as described in the text. A Student's t-test was applied to the raw data; results are expressed as means±s.e.m. of n cells (except where otherwise stated) with a value of \(P<0.05\) being considered significant.

**Results**

The response of intact strips of distal colon to carbachol

Carbachol (CCh, 0.5 \(\mu M\)) produced an initial large increase in force (phasic component) followed by a decline to a more sustained level (tonic component, Fig. 1A). To examine its role in these components, external Ca\(^{2+}\) was removed from the bathing solution. Under these circumstances, the phasic component to CCh remained, although reduced (10.9±0.6 g vs 7.5±0.7 g in Ca\(^{2+}\)-free external solution, \(n=17\) tissues), while the tonic component was almost abolished (6.3±0.4 g vs 1.5±0.8 g, \(n=17\) tissues; Fig. 1A). To determine the contribution of Ins\(P_3\), to the phasic and tonic components, the Ins\(P_3\) receptor blocker 2-APB (100 \(\mu M\)) was used (Ascher-Landsberg et al., 1999). Unexpectedly, 2-APB reduced both phasic (7.6±0.9 g vs 2.4±0.8 g) and tonic components (4.3±0.7 g vs 2.5±0.7 g, \(n=8\) tissues; Fig. 1B) significantly (\(P<0.05\)). Both components recovered on washout of 2-APB (phasic to 7.4±1.0 g, tonic to 5.9±1.0 g, \(n=8\) tissues; Fig. 1B).

2-APB, in addition to blocking Ins\(P_3\) receptors, may also inhibit Ca\(^{2+}\) entry via store-operated channels. This might have accounted for the reduction in the tonic component (Gregory et al., 2001). However, the store-operated channel blocker SKF 96365 (10 \(\mu M\)) did not reduce the tonic component (control tonic, 7.3±0.8 g; after SKF 96365, 7.2±0.9 g, \(P>0.05\), \(n=6\) tissues, data not shown) suggesting that the reduction of the tonic component by 2-APB may be dependent on Ins\(P_3\) receptor blockade rather than on blockade of store-operated Ca\(^{2+}\) entry. Indeed, the tonic contractile component required Ca\(^{2+}\) entry via voltage-dependent Ca\(^{2+}\) channels rather than via store-operated channels, since the tonic component was blocked by nimodipine (50 nM), a dihydropyridine inhibitor of voltage-dependent Ca\(^{2+}\) channels (control tonic, 5.4±0.9 g; after nimodipine, 1.4±0.4 g, \(n=13\) tissues, \(P<0.05\), data not shown). In contrast, nimodipine had a less (but significant) effect on the phasic component [control 9.4±1.2 g and 4.0±0.6 g after nimodipine (50 nM), \(n=13\) tissues, \(P<0.05\), data not shown].

The nature of the periodic outward currents

The results suggested that Ins\(P_3\) receptors could be involved in both phasic and tonic components and prompted an investigation of the basis of this involvement. Ca\(^{2+}\) release from stores reportedly regulates contraction via spontaneous transient outward currents [STOCs (Nelson et al., 1995)]. Accordingly, to explore the possibility that modulation of STOCs may form the basis of Ins\(P_3\) effects on contraction, single smooth muscle cells were isolated and the effects of Ins\(P_3\)-inducing agonists on STOCs examined using patch clamp techniques. Depolarisation to between –20 mV and 0 mV, from a holding potential of –70 mV, increased [Ca\(^{2+}\)], and activated periodic outward currents (Fig. 2Ai,ii) which increased in frequency and amplitude over several seconds even as [Ca\(^{2+}\)] declined (Fig. 2Aiv). Currents varied widely in amplitude, frequency and duration (expanded time base Fig. 2Ai, and Fig. 2Aiii, which is an ‘all-points histogram’ of the membrane current recording at –20 mV). The mean outward current amplitude (±s.e.m.) was 116±30 pA, the rise time 19±13 milliseconds, the \(t_{0.5}\) of decay 26±7 milliseconds and the \(t_{0.9}\) of decay 36±1 milliseconds (\(n=758\) from three cells). The periodic outward currents were indeed STOCs (Benham and Bolton, 1986); they were inhibited, in separate experiments, each by the potassium channel blocker TEA (20 mM, Fig. 2Bi) and by ryanodine (50 \(\mu M\), Fig. 2Ci). Thus, before TEA, STOCs produced a charge entry of 123±32 pC whereas, after TEA, this had declined to –1±5 pC (\(n=3\), \(P<0.05\), 5 second integral). Before ryanodine (50 \(\mu M\)), the 5 second integral was 47±8 pC; after the drug, this was 3±5 pC (\(n=3\), \(P<0.05\)).

**Effects of Ins\(P_3\), caffeine and CCh on STOCs**

Depolarisation from –70 mV to –10 mV elevated [Ca\(^{2+}\)], and activated STOCs (Fig. 3i-iv). Ins\(P_3\), caffeine (10 mM) and CCh
(50 μM) each transiently increased [Ca²⁺], and reversibly inhibited STOCs. In six identical experiments, a 5 second integral of the membrane current decreased from 143±33 pC to 9±14 pC (P<0.05) after InsP₃, from 105±24 pC to –20±15 pC (P<0.01) after caffeine, and from 86±15 pC to –33±17 pC (P<0.05) after CCh. CCh suppression of STOCs was reproducible, being seen in each of 42 cells examined and up to five times in the same cell. The first CCh application increased [Ca²⁺] (by 1.0±0.2 ΔF/F₀ units above baseline, n=5) and suppressed STOCs to 4±2% of their pre CCh value (89±21 pC vs 3±2 pC, n=5, P<0.05). The second application of CCh also increased [Ca²⁺], (by 0.9±0.2 ΔF/F₀, n=5) and suppressed STOCs to –2±3% of their pre CCh value (56±7 pC to –1±9 pC, n=5, P<0.05). Both the CCh-evoked increase in [Ca²⁺], and the suppression of STOCs were blocked by atropine (10 μM); the [Ca²⁺] increase evoked by CCh was reduced by 97% to 3±3% of controls in the same cells (1.4±0.6 ΔF/F₀ vs 0.1±0.1 ΔF/F₀, n=3, P<0.05) and CCh suppression of STOCs was reduced. In controls, STOCs were reduced to 12±12% of their value by CCh (99±35 pC vs 15±14 pC, n=3, P<0.05). After atropine, in these same cells, the extent of the suppression of STOCs by CCh had been reduced and they remained at 77±17% of their control value (85±21 pC vs 73±28 pC, n=3, P>0.05).

Caffeine suppressed STOCs presumably by activating RyR, thus depleting the RyR-sensitive store of Ca²⁺ (e.g. Bolton and Lim, 1989; Ganitkevich and Isenberg, 1995); InsP₃ may also deplete the RyR-sensitive store of Ca²⁺, but via a different route. To explore this possibility, the effects of ryanodine on InsP₃-evoked Ca²⁺ release were examined. Ryanodine, significantly reduced the InsP₃-evoked Ca²⁺ transient to 68±15% of control values (P<0.05, n=8; Fig. 4). InsP₃ increased [Ca²⁺], by 1.9±0.3 ΔF/F₀ units above baseline under control conditions (the sixth control InsP₃ release; n=8); the sixth InsP₃-mediated Ca²⁺ release in the presence of ryanodine (50 μM; 1.3±0.4 ΔF/F₀ units above baseline, n=8, P<0.05) was significantly less than in the absence of the drug. In the same experiment, activation of RyR by caffeine both increased [Ca²⁺], and apparently depleted the store of Ca²⁺ so that a second caffeine application failed to increase [Ca²⁺], (by 0.07±0.02 ΔF/F₀ units above baseline, n=8, P<0.05; Fig. 4). Significantly, after the second caffeine application, InsP₃ no longer evoked Ca²⁺ release. This latter finding suggests that the InsP₃ receptor and RyR have access to a common Ca²⁺ store.
(Flynn et al., 2001), thus InsP$_3$ receptor activity could inhibit STOCs by reducing the Ca$^{2+}$ available for release via the RyR.

The extent to which the store’s Ca$^{2+}$ content had to be reduced to inhibit STOCs was next examined. The store’s Ca$^{2+}$ content was assessed from the amplitude of InsP$_3$-evoked transients and compared with the probability of STOC occurrence ($P_{STOC}$) as the store’s content decreased. The store was depleted of Ca$^{2+}$ by incubating cells in a Ca$^{2+}$-free solution [containing 3 mM MgCl$_2$ and 1 mM EGTA (McCarron et al., 2000)]. Ca$^{2+}$-entry induced Ca$^{2+}$ release, from the RyR, plays a minor role in the generation of STOCs since the currents persist for some time in the presence of Ca$^{2+}$ channel blockers such as cadmium (Benham and Bolton, 1986; Nelson et al., 1995). The time course of disappearance of STOCs (at –10 mV) in the Ca$^{2+}$-free solution was first examined, then the time course of reduction of InsP$_3$ store content (as indicated by the amplitude of Ca$^{2+}$ transients) at various times (30 seconds to 8 minutes) was investigated. Both STOCs and InsP$_3$-evoked Ca$^{2+}$ transients were abolished in the Ca$^{2+}$ free solution (Fig. 5). STOCs were the more sensitive. The probability of STOC occurrence was reduced by 70% from 0.3±0.05 to 0.09±0.025 after 30 seconds in Ca$^{2+}$-free solution. At the same time (30 seconds), the InsP$_3$-sensitive Ca$^{2+}$ store content was reduced by only 16±7% of control values as assessed by the magnitude of the InsP$_3$-evoked Ca$^{2+}$ transient (Fig. 5iv). The store therefore seems to require a substantial Ca$^{2+}$ load to generate STOCs. Together (Figs 4, 5), these results suggest that a relatively modest reduction in SR Ca$^{2+}$ content is required to inhibit STOCs.

The contribution of CICR, at the RyR, to InsP$_3$-evoked increases in [Ca$^{2+}$]

The finding that ryanodine, by itself, decreased the InsP$_3$-evoked Ca$^{2+}$ transient (Fig. 4) may be explained in two ways. First, InsP$_3$-evoked Ca$^{2+}$ release could have triggered CICR at the RyR; in the presence of ryanodine this would be prevented so that the Ca$^{2+}$ transient evoked by InsP$_3$ would appear reduced. Secondly, by locking the RyR into a subconductance level (Smith et al., 1988; Anderson et al., 1989; Xu et al., 1994), ryanodine may have rendered the store leaky, reducing both the total SR Ca$^{2+}$ content and that available to InsP$_3$. To distinguish between these possibilities, tetracaine (100 μM), a local anaesthetic with RyR blocking activity, was used to inhibit RyR (Pizarro et al., 1992; Gyorke et al., 1997). Tetracaine did not reduce the InsP$_3$-evoked Ca$^{2+}$ transient (n=8) but inhibited STOCs (300±61 pC vs 72±23 pC, 20 second integrals, n=5, P<0.05), a finding consistent with its inhibitory action on the RyR (Fig. 6A,B). The latter result could not be explained by an inhibitory action of tetracaine on the Ca$^{2+}$-activated K$^+$ channel itself since the peak Ca$^{2+}$-activated K$^+$ current activated by InsP$_3$-evoked Ca$^{2+}$ release was unaltered by the drug (740±91 pA vs 679±81 pA in the presence of tetracaine, n=6, P>0.05, data not shown). The reduction in the InsP$_3$-evoked Ca$^{2+}$ transient by ryanodine alone (Fig. 4), probably occurred as a result of the drug’s rendering the SR leaky to Ca$^{2+}$ so reducing its Ca$^{2+}$ content. Collectively, these experiments (Fig. 6A,B) suggest that: (1) InsP$_3$ did not activate CICR at the RyR (had it done so, the amplitude of the InsP$_3$-evoked Ca$^{2+}$ transient would have been decreased by tetracaine); and (2) STOCs arise from the RyR and not the InsP$_3$ receptor (since tetracaine blocked STOCs while leaving the InsP$_3$-evoked Ca$^{2+}$ transient unaffected).

The contribution of InsP$_3$ and protein kinase C to CCh-evoked suppression of STOCs

CCh suppression of STOCs could have arisen from its ability to produce InsP$_3$ and so deplete the SR Ca$^{2+}$ store. If so,

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**Fig. 3.** The effects of InsP$_3$, caffeine and carbachol (CCh) on STOCs. Depolarisation from –69 mV to –10 mV (iv) elevated [Ca$^{2+}$]$_i$ (iii) and activated STOCs (ii). InsP$_3$ (†), caffeine (Caff, 10 mM, v) and CCh (50 μM) each increased [Ca$^{2+}$]$_i$ (iii) and reversibly inhibited STOCs (ii and expanded time scale i).
blocking the InsP3 receptor with, for example, 2-APB, should prevent the suppression. This proved to be the case. The increase in \([\text{Ca}^{2+}]_i\) evoked by CCh was significantly (\(P<0.05\)) reduced by 2-APB to 23±14% of control values (by 0.9±0.14 \(\Delta F/F_0\) vs 0.2±0.14 \(\Delta F/F_0\), \(n=6\); Fig. 7). CCh by itself reduced STOCs to 9±14% of their pre-CCh value (145±31 pC vs 13±12 pC, \(n=6\), \(P<0.05\)). After 2-APB (50 \(\mu\)M), in these same cells, CCh-suppression of STOCs was reduced and the currents remained at 85±19% of their pre-CCh value (110±29 pC vs 94±33 pC, \(n=6\), \(P<0.05\); Fig. 7). Similar results were obtained

Fig. 4. The effects of ryanodine on InsP3-evoked \([\text{Ca}^{2+}]_i\) transients and STOCs. At a holding potential of –20 mV (iv) InsP3 (†) increased \([\text{Ca}^{2+}]_i\), (ii); ryanodine (50 \(\mu\)M) significantly reduced the InsP3-evoked \([\text{Ca}^{2+}]_i\) transients (i,ii). Activation of RyR by caffeine (Caff, 10 mM, iii) increased \([\text{Ca}^{2+}]_i\) (ii). A second application of Caff some 60 seconds later almost abolished both the \([\text{Ca}^{2+}]_i\) transient, presumably by depleting the SR store and the InsP3 response (†) leaving only the artefact (ii). Because the InsP3-evoked \([\text{Ca}^{2+}]_i\) transient was blocked after caffeine in the presence of ryanodine, InsP3 receptors and RyR may share a common \([\text{Ca}^{2+}]_i\) store. i is a summary of eight experiments.

Fig. 5. Effects of withdrawal of extracellular \([\text{Ca}^{2+}]_i\) on the rate of decline of STOCs and on the response to InsP3. Depolarisation to –10 mV from a holding potential of –70 mV (iii) induced STOCs (i) and raised \([\text{Ca}^{2+}]_i\), (ii). Removal of extracellular \([\text{Ca}^{2+}]_i\) for the duration indicated by the bar abolished STOCs and lowered \([\text{Ca}^{2+}]_i\) to pre-depolarisation levels. The times on the bars indicate the period in \([\text{Ca}^{2+}]_i\)-free/1mM EGTA solution prior to the release of InsP3 (ii). A 4 minute period separated the traces as indicated by the gap. At –10 mV the responses to InsP3 (†, iii) were elicited 0.5, 1, 2, 4 and 8 minutes after \([\text{Ca}^{2+}]_i\) withdrawal and compared with control responses to InsP3 obtained before removal of extracellular \([\text{Ca}^{2+}]_i\). \([\text{Ca}^{2+}]_i\) withdrawal reduced the amplitude of both STOCs and the InsP3 responses, the rate of decline of the former exceeded that of the latter (iv). These results suggest that the \([\text{Ca}^{2+}]_i\) store content required to support STOCs is greater than that to maintain InsP3 responses.
with the impermeable InsP$_3$ receptor inhibitor heparin. After heparin (2.5 mg/ml, ~15 minutes), the $[\text{Ca}^{2+}]_i$ increase in response to InsP$_3$ was insignificant: 0.03±0.02 ΔF/F$_0$ units above baseline (resting 1.3±0.1 F/F$_0$ vs 1.4±0.1 F/F$_0$ after InsP$_3$, n=5, P>0.05); nor were STOCs significantly different in either frequency or amplitude (126±53 pC vs 126±48 pC after InsP$_3$, n=5, P>0.05) from controls. In these same cells, the CCh (50 μM)-evoked $[\text{Ca}^{2+}]_i$ increase in the presence of heparin was, like the InsP$_3$ response, attenuated to 0.2±0.04 ΔF/F$_0$ units above baseline so that the rise in $[\text{Ca}^{2+}]_i$ was insignificant (from a resting value of 1.3±0.1 F/F$_0$ in the absence of CCh to 1.6±0.2 F/F$_0$ after CCh, n=5, P>0.05) as was the suppression of STOCs (106±40 pC before and 88±35 pC after CCh, n=5, P>0.05). Together these results indicate that the InsP$_3$ receptor is essential for the CCh-evoked suppression of STOCs and that this mechanism underlies the depletion of the SR Ca$^{2+}$ store by the agonist.

Other second messengers, such as protein kinase C (PKC) may also have contributed to the inhibition of STOCs by mechanisms other than by changes in either InsP$_3$ or the SR Ca$^{2+}$ store content. To examine this possibility, the effect of the broad spectrum protein kinase inhibitor H-7 (10 μM) was examined. H-7 did not reduce the ability of CCh to inhibit STOCs (Fig. 8A). Under control conditions, at depolarised membrane potentials (~–20 mV), STOCs produced an integrated current of 103±44 pC, which was reduced to –3±2 pC after CCh (n=7, P<0.05). In the presence of H-7, perfused some 10 minutes beforehand, STOCs produced an integrated current of 98±30 pC whereas, after CCh, the charge entry was reduced to 2±3 pC (n=7, P<0.05; Fig. 8A). Other protein kinase C inhibitors were no more effective. After the protein kinase C inhibitory peptide (PKC19-36, 3 mM, ~10 minutes; Fig. 8B), InsP$_3$ and CCh each inhibited STOCs. Before InsP$_3$, STOCs evoked a 5 second integrated current of 107±27 pC whereas, after InsP$_3$, the charge entry was reduced to –6±1.2 pC (n=3, P<0.05; Fig. 8B). Other protein kinase C inhibitors were no more effective. After the protein kinase C inhibitory peptide (PKC19-36, 3 mM, ~10 minutes; Fig. 8B), InsP$_3$ and CCh each inhibited STOCs. Before InsP$_3$, STOCs evoked a 5 second integrated current of 107±27 pC whereas, after InsP$_3$, the charge entry was reduced to –6±1.2 pC (n=3, P<0.05; Fig. 8B). Charge entry before CCh was 64±14 pC and, after CCh, was –32±18 pC (n=3, P<0.05). These results indicate that CCh suppression of STOCs is independent of protein kinase C.

The latter finding may occur because protein kinase C cannot suppress STOCs or, alternatively, because the effect of CCh on STOCs is not mediated via the kinase. To distinguish between these possibilities the protein kinase C activator indolactam was used. Indolactam (10 μM) significantly inhibited STOCs (Fig. 8C). Before indolactam, $[\text{Ca}^{2+}]_i$ was 1.6±0.2 ΔF/F$_0$ units and STOCs was 386±62 pC (20 second integrated current) whereas, after indolactam, $[\text{Ca}^{2+}]_i$ was 1.4±0.1 ΔF/F$_0$ units (P>0.05) and STOCs was 161±51 pC (20 second integral, P<0.05, n=9 in all cases). The inhibitory action of indolactam (10 μM) on STOCs was fully blocked by the inhibitory peptide PKC19-36 (3 mM) introduced into the cell via the patch pipette (304±85 pC before and 395±152 pC after indolactam, n=6, 20 second integrals). Together, these results indicate that while protein kinase C activation can suppress STOCs, muscarinic receptor activation with CCh does not suppress STOCs by activating the kinase.

**Discussion**

The present study has shown that InsP$_3$ contributes to both the phasic and tonic components of muscarinic-induced smooth muscle contraction. Each component was inhibited by InsP$_3$ receptor blockade. Since the tonic contractile component is dependent on entry via voltage-dependent Ca$^{2+}$ channels, InsP$_3$ is modulating Ca$^{2+}$ entry via these channels. One explanation for these findings is that muscarinic receptor-generated InsP$_3$ evokes a transient Ca$^{2+}$ release from the Ca$^{2+}$ store that then evokes the phasic component. Depletion of this store suppresses STOCs since the store responsible for them is accessed by both RyR and InsP$_3$ receptors. STOC suppression
could, in turn, lead to depolarisation and a sustained Ca\(^{2+}\) entry via voltage-dependent Ca\(^{2+}\) channels in the sarcolemma giving rise to the tonic component. Indeed, support for the involvement of Ins\(_{P3}\) receptors in the tonic response to muscarinic activation is already evident from the observation that, in certain murine smooth muscles lacking the type 1 Ins\(_{P3}\) receptor, cholinergic depolarisation is impaired (Suzuki et al., 2000). The proposed scheme differs from that in non-excitable cells where depletion of the store generates a sustained elevation in Ca\(^{2+}\) as a consequence of entry via the voltage-independent store-operated channels. If such a mechanism were operative in excitable cells, the depolarisation accompanying agonist activation would reduce Ca\(^{2+}\) via this route [because of a reduced driving force on the ion (see McCarron et al., 2000)] compromising the sustained response. In the present proposal, the depolarisation, caused by the suppression of STOCs, forms a central part of the response and is generated by store depletion.

STOCs arise from the Ca\(^{2+}\) store, access to which is shared by RyR and the Ins\(_{P3}\) receptors. Suppression of STOCs is a direct consequence of depletion of Ca\(^{2+}\) in this store by Ins\(_{P3}\). Ins\(_{P3}\) did not, for example, deplete a separate store by releasing Ca\(^{2+}\) and activating CICR at the RyR; tetracaine, which blocks RyR, had no effect on the Ins\(_{P3}\)-evoked Ca\(^{2+}\) transient. Furthermore, at negative sarcolemma potentials (~–70 mV), where RyR activity is reduced (Jaggar et al., 1998), ryanodine had no effect on the Ins\(_{P3}\)-evoked Ca\(^{2+}\) transient (Flynn et al., 2001). We have previously proposed the existence of two SR Ca\(^{2+}\) stores in these cells (Flynn et al., 2001), one containing both Ins\(_{P3}\) receptors and RyR, the other containing RyR alone. Since block of the RyR did not affect the Ins\(_{P3}\)-evoked Ca\(^{2+}\) transient, only the store with both receptors was responsible for the generation of STOCs (Bolton and Lim, 1989).

The finding that both Ins\(_{P3}\) receptors and RyR have access to a common Ca\(^{2+}\) store is based partly on the conclusion that Ins\(_{P3}\)-evoked Ca\(^{2+}\) release did not activate CICR at the RyR. Notwithstanding, ryanodine reduced the Ins\(_{P3}\)-evoked Ca\(^{2+}\) transient in the present study at depolarised membrane potentials (~–20 mV), appearing to indicate that Ins\(_{P3}\)-evoked release activated CICR at the RyR. An alternative explanation to CICR involvement is that ryanodine, by maintaining the RyR in an open configuration could have attenuated the Ins\(_{P3}\)-evoked Ca\(^{2+}\) transient by reducing the SR Ca\(^{2+}\) content. In support, tetracaine, which does not open but blocks the RyR, did not reduce the Ins\(_{P3}\)-evoked Ca\(^{2+}\) transient. At membrane potentials of ~–20 mV (used in this study) the RyR are active, as shown by their electrical manifestation at the sarcolemma (i.e. STOCs). Ryanodine binds to the open state of the RyR (Meissner and El-hashem, 1992; McPherson and Campbell, 1993; Ogawa, 1994) and, in the concentration range used in the present study, may prolong its open time of the receptor albeit at a lower conductance (Xu et al., 1994). [Higher concentrations of ryanodine than those presently used [e.g. 300 µM (Janiak et al., 2001)] may stabilise the channel in the closed state.] The persistent opening of the RyR would

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**Fig. 7.** The effects of Ins\(_{P3}\) receptor blockade on the ability of carbachol (CCh) to suppress STOCs. Depolarisation to ~–20 mV from a holding potential of ~–70 mV (iv) induced STOCs (ii) and increased [Ca\(^{2+}\)], (iii). (A) 2-APB (50 µM), a membrane-permeable Ins\(_{P3}\) receptor inhibitor, introduced by perfusion, inhibited the ability of Ins\(_{P3}\) and CCh to affect STOCs (ii and i expanded time base). (i) represents some 30 second excerpts from ii as indicated by the dotted lines. The increased perfusion per se temporarily increased STOC amplitude and was unrelated to the presence of a particular drug. (B) Heparin (2.5 mg/ml), a membrane-impermeable Ins\(_{P3}\) receptor inhibitor, introduced via the patch pipette, was present throughout the entire experiment. Other experiments (not shown) under identical conditions with no heparin present served as controls. In the presence of heparin, neither Ins\(_{P3}\) (†) nor CCh (10 mM, v) significantly altered the amplitude or frequency (ii and i expanded time base i) of STOCs. CCh activated a transient inward current causing the resting level of membrane current to fall (ii).
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increase Ca²⁺ leak from the SR, lower its Ca²⁺ content and thus reduce the InsP₃-evoked Ca²⁺ transient. Consistent with this scheme, at a membrane potential of −70 mV, ryanodine did not alter the InsP₃-evoked Ca²⁺ transient (Flynn et al., 2001). RyR, in smooth muscle, is less active at negative membrane potentials (e.g. −70 mV) and increases with depolarisation presumably reflecting increasing [Ca²⁺]i and/or voltage-dependent Ca²⁺ channel activity (Jaggar et al., 1998). Because of the reduced opening of the RyR, ryanodine will be less effective and would not be expected to reduce the InsP₃-evoked Ca²⁺ transient. Together these results provide the evidence that Ca²⁺ release from the InsP₃ receptor does not activate CICR at the RyR but that ryanodine reduces the InsP₃-evoked Ca²⁺ transient by increasing Ca²⁺ leak from the store.

The conclusion that InsP₃-evoked Ca²⁺ release does not trigger CICR at the RyR disagrees with that of others in which the reduction of the Ca²⁺ transient evoked by InsP₃-generating agents, by ryanodine, was interpreted as evidence that InsP₃-evoked Ca²⁺ activates CICR at the RyR (Boittin et al., 1999; Jaggar and Nelson, 2000). The sarcolemma agonists used in these latter studies to generate InsP₃ (as opposed to caged InsP₃ used in the present study) may also have activated other second messengers that sensitized the RyR to Ca²⁺.

Alternatively, since the ability of Ca²⁺ release to activate CICR at neighbouring RyR increases with SR Ca²⁺ content, release may activate further release under conditions of 'store overload' (Cheng et al., 1996; Trafford et al., 1995). Some smooth muscle types may maintain a higher SR Ca²⁺ content facilitating CICR at the RyR.

The mechanism by which muscarinic receptor activation suppresses STOCs was deduced from studies on the effects of the muscarinic agonist CCh. CCh suppressed STOCs; this inhibition was prevented by each of the InsP₃ receptor blockers 2-APB and heparin but not by the protein kinase C inhibitors H-7 or PKC19-36. Photolyzed caged InsP₃ also suppressed STOCs, confirming the view that the ability of CCh to suppress STOCs is solely dependent on the production of InsP₃ (see also Komori and Bolton, 1990). These present results do not preclude the involvement, in other smooth muscles, of other second messenger systems where other neurotransmitters may be operative [e.g. in rabbit portal vein (Kitamura et al., 1992) and rat cerebral artery (Jaggar and Nelson, 2000)]. In murine colonic myocytes, muscarinic receptor activation was reported to inhibit STOCs by increasing the bulk average [Ca²⁺]i,
of the stores presumably reflected the time treatments used to elevate $[\text{Ca}^{2+}]_i$ (e.g. ionomycin or ACh).

P

The suppression of STOCs; STOC inhibition by CCh or InsP$_3$ oscillations (Duncan et al., 1987; Hirose et al., 1999). Significantly, InsP$_3$ desensitisation, metabolism of the neurotransmitter or oscillate after receptor activation as a result of receptor minutes after CCh washout. In other tissues, the levels of InsP$_3$ (Salmon and Bolton, 1988), consistent with present this is the case. InsP$_3$ through the presence of the agonist. There is evidence that depend on InsP$_3$ content and STOC occurrence (ZhuGe et al., 1999).

If both agonist-induced phasic and tonic components each depend on InsP$_3$ production, this substance must be available throughout the presence of the agonist. There is evidence that this is the case. InsP$_3$ formation, as deduced both from the disappearance of its precursor phosphatidylinositol 4,5-bisphosphate and by direct measurement of InsP$_3$ itself, is indeed sustained throughout the period of agonist stimulation up to 1 hour (Akhtar and Abdel-Latif, 1984; Baron et al., 1984; Takuwa et al., 1986; Marc et al., 1988). For example, in guinea-pig intestinal smooth muscle stimulated by CCh for 1 minute, elevated InsP$_3$ levels were detected for more than 5 minutes (Salmon and Bolton, 1988), consistent with present observations where STOCs remained inhibited for periods of minutes after CCh washout. In other tissues, the levels of InsP$_3$ oscillate after receptor activation as a result of receptor desensitisation, metabolism of the neurotransmitter or feedback regulation of production. Significantly, InsP$_3$ concentration is maintained above resting levels during these oscillations (Duncan et al., 1987; Hirose et al., 1999).

The present results demonstrate that activation of muscarinic receptors on smooth muscle evokes a tonic contraction by the generation of InsP$_3$. However, such a result raises the controversial issue of whether or not the neurotransmitter itself directly contacts the smooth muscle cell. Recent evidence has proposed that interstitial cells of Cajal (ICCs) serve as intermediate transducers of the nerve response in certain smooth muscles. Reduction or elimination of these cells abolished both inhibitory (nitrergic) and excitatory (cholinergic) transmission (Ward et al., 1998; Ward et al., 2000). Others have failed to repeat these findings and found neurotransmission unimpaired in preparations from mice lacking ICCs (Sivarao et al., 2001). Since some extrinsic nerves make close synaptic contact (20 nm) with smooth muscle as well as innervating ICCs, neural transmission may persist in the absence of ICCs (FaussonePellegrini et al., 1989). A difficulty, with the use of mice lacking ICCs, is that differences in smooth muscle contractility exist that are unrelated to the innervation (Sivarao et al., 2001). Thus stomachs of the ICC-deficient mice lack basal tone and are more compliant than the corresponding atropine-treated controls (Ward et al., 2000). The origin of such differences must await further investigation.

Agonist activation evokes smooth muscle contraction via the activation of several signalling systems which include activation of non-capacitative $\text{Ca}^{2+}$ entry pathways (Broad et al., 1999) such as cationic channels (Pacaud and Bolton, 1991; Zholos and Bolton, 1997), alterations in the myofilament $\text{Ca}^{2+}$ sensitivity (Somlyo and Somlyo, 2000) and $\text{Ca}^{2+}$ release from the internal stores (Sims et al., 1997). The present results reveal that the traditionally recognised phasic and tonic components of agonist-induced smooth muscle contraction may be mediated, at least in part, by InsP$_3$. The mechanism proposed helps to explain why excitatory G-protein-coupled agonists, such as ACh, triggered biphasic changes in both $[\text{Ca}^{2+}]_i$ and the contractile state (Himpens and Somlyo, 1985; Williams and Fay, 1986). The first component is transient, reflecting $\text{Ca}^{2+}$ release from the internal store. In the second (tonic component), depletion of the store leads to $\text{Ca}^{2+}$ entry via voltage-dependent $\text{Ca}^{2+}$ channels because of the depolarisation that arises from the suppression of STOCs, which generates a tonic contractile phase; each component requires InsP$_3$. The authors thank Stuart Cobbe for useful discussions. This work was funded by the Wellcome Trust (055432/Z/98/Z) and British Heart Foundation (PG/2001079). J.G.M. was a Caledonian Research Foundation Fellow when this work was carried out.

References


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