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Uterine Contractility Symposium

Receptor-coupled contractility of uterine smooth muscle: from membrane to myofilaments

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A comprehensive understanding of the mechanisms by which agonists control uterine contraction is essential for the successful clinical management of parturition and for the timely treatment of situations involving inappropriate uterine performance. In this review we discuss some of the key stimulatory mechanisms linking receptor occupation at the myometrial plasma membrane with alteration of myofilament activation. We focus on evidence that receptor-induced membranous recruitment of the small G-protein rhoA, and its downstream effector rho-associated kinase (ROK) is crucial to agonist-induced Ca^{2+}-sensitisation of uterine contraction and that co-ordination of this signal transduction pathway may be mediated by the actions of caveolins, proteins integral to specialised membranous regions termed caveolae. *Experimental Physiology* (2001) **86.2**, 283–288.

Agonist-stimulated contraction of smooth muscle, including that of the uterus, involves the integration of many signal transducing events linking receptor occupation at the plasma membrane with varied intracellular effectors and eventual alteration of the activation state of the myofilaments in the cell cytoplasm. Precise co-ordination of these myometrial signalling events at term is paramount to ensuring efficient and powerful contractile activity necessary for expeditious expulsion of the fetus. A comprehension of the complex intracellular mechanisms of action of uterotonic agents is thus essential not only to our understanding of parturition but also for the management of situations involving dysfunctional uterine performance including, for example, those associated with pre-term labour, non-labouring uteri and post-partum haemorrhage. As such, in this paper we review recent studies that shed light on many of the mechanisms contributing to the efficient coupling of extracellular contractile stimuli and intracellular effectors in uterine smooth muscle. Special consideration is given to the processes of receptor-coupled recruitment to the plasma membrane of intracellular proteins important for agonist-induced alterations in Ca^{2+} sensitivity of contraction such as rhoA and ROK (rho-associated kinase); evidence for the involvement of caveolins, proteins integral to plasma membranous caveolae, in this signal transduction cascade is discussed.

Receptor activation of uterine smooth muscle

Agonist-stimulation of uterine smooth muscle primarily involves an elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) via increased trans-sarcolemmal influx and/or release of Ca^{2+} from intracellular stores especially those responsive to generation of IP_3 (inositol 1,4,5-trisphosphate; Taggart & Wray, 1998). The elevation in $[Ca^{2+}]_i$ results in activation of the Ca^{2+}- and calmodulin-dependent enzyme myosin light chain kinase and subsequent phosphorylation of the regulatory myosin light chains (MLC_{20}; Word *et al.* 1991; Taggart *et al.* 1997). This increased phosphorylation of MLC_{20} allows for enhanced actomyosin Mg-ATPase activity and subsequent contractility. Smooth muscle relaxation is preceded by dephosphorylation of MLC_{20} by a myosin phosphatase (MLCP; Khromov *et al.* 1995) which has been cloned from uterine tissue (Johnson *et al.* 1997). However, a prominent additional mechanism whereby agonists can stimulate smooth muscle contractility is by sensitising the myofilaments to the activating $[Ca^{2+}]_i$. This occurs in both intact (Taggart & Wray, 1998) and permeabilised (Izumi *et al.* 1996; Somlyo & Somlyo, 1998; Taggart *et al.* 1999) uterine smooth muscle and, in other smooth muscles, has been associated with corresponding elevations of MLC_{20} phosphorylation

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(Kitizawa *et al.* 1991; Fu *et al.* 1998; Swärd *et al.* 2000). This agonist-induced Ca^{2+} sensitisation of MLC_{20} phosphorylation and force is thought to result from inhibition of MLCP (Kureishi *et al.* 1996; Swärd *et al.* 2000) although an involvement of thin filament-linked regulation has also been suggested for other smooth muscles (Horowitz *et al.* 1996).

Agonist Ca^{2+} sensitisation of single uterine smooth muscle cell force production

As first reported by Taggart *et al.* (1999), and illustrated in Fig. 1, the contractile force of permeabilised single isolated uterine smooth muscle cells from pregnant rat can be monitored in response to agonist application. Brief permeabilisation of these cells with saponin allows the concentration of activating Ca^{2+} surrounding the myofilaments to be strictly controlled by that in the bathing milieu. At suprabasal, but sub-maximal activating Ca^{2+} (pCa 6.7; Crichton *et al.* 1993) application of the muscarinic agonist carbachol results in pronounced contraction of these single uterine cells. Carbachol application resulted in contractions of magnitude $4.30 \pm 0.30\ \mu N$ ($n = 5$) similar to that reported previously for uterine smooth muscle (Taggart *et al.* 1999) but significantly greater than that observed for agonist stimulation of isolated vascular smooth muscle cells (Collins *et al.* 1992; Lee *et al.* 1999). Wortmannin, an inhibitor of myosin light chain kinase (MLCK), completely reversed carbachol-induced Ca^{2+} sensitisation ($n = 5$) – and even slightly decreased the basal force in pCa 6.7 (Fig. 1) – indicating an important role of an active MLCK to the sensitisation process. It is to be noted, however, that wortmannin is also capable of inhibiting several other kinases (Linseman *et al.* 1999; Duan *et al.* 2000).

One molecule suggested to be important for agonist-induced Ca^{2+} sensitisation is the small G-protein rhoA which acts downstream of receptor occupancy. Inactivation of rhoA in a variety of smooth muscles by ADP-ribosylation (Otto *et al.* 1996; Fujihara *et al.* 1997) or monoglucosylation (Otto *et al.* 1996; Lucius *et al.* 1998) results in inhibition of Ca^{2+} sensitisation. The Ca^{2+}-sensitising effects of rhoA activation appear mediated, in part at least, by its effector molecule ROK. As shown in Fig. 1, pharmacological inhibition of ROK with Y-27632 (Ueheta *et al.* 1997) results in $91 \pm 5.6\%$ ($n = 4$) reduction of carbachol-induced Ca^{2+} sensitisation of single uterine smooth muscle cell force. This is in agreement with the abrogation of oxytocin-induced Ca^{2+} sensitisation in multicellular preparations (Somlyo & Somlyo, 1998) indicating that multiple uterotonic agents may sensitise the myofilaments to Ca^{2+} via a rhoA/ROK-mediated reduction of MLCP activity. Notably, uterine ROK expression is increased with pregnancy (Niiro *et al.* 1997; Moore *et al.* 2000) as is agonist-induced Ca^{2+} sensitisation of force (Taggart & Wray, 1998). However, the assumed inhibition of MLCP activity in uterine single smooth muscle cells by activation of ROK, at least in response to muscarinic activation, is unlikely to be complete as application of the phosphatase inhibitor microcystin increases Ca^{2+} sensitisation to carbachol by a further $60.5 \pm 12.4\%$ ($n = 3$; see Fig. 1). Additionally, in light of these results and also the rather steep

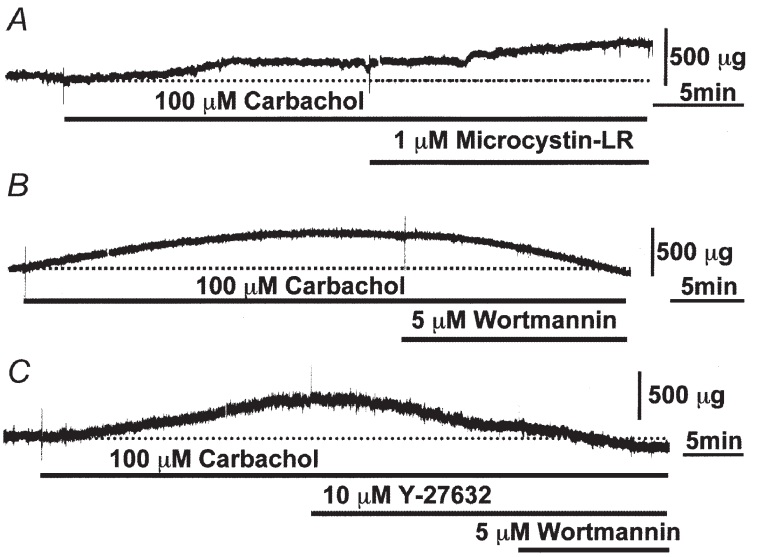

Figure 1

Agonist-induced Ca^{2+} sensitisation of single uterine smooth muscle cell force production. Single uterine smooth muscle cells were enzymatically isolated from 19-day pregnant rat, briefly permeabilised with saponin and prepared for measurement of force as described by Taggart *et al.* (1999). Permeabilised cells incubated in pCa 6.7 solution exhibited prominent agonist-induced Ca^{2+} sensitisation by contracting strongly to 100 μM carbachol (CCh). *A*, enhancement of CCh-induced Ca^{2+}-sensitised contractions by the phosphatase inhibitor microcystin-LR. *B*, inhibition of agonist-induced contractions by MLCK inhibitor wortmannin. *C*, reduction of CCh-induced contractions by ROK inhibitor Y-27632.

relationship reported between agonist Ca^{2+} sensitisation of MLC_{20} phosphorylation and force in smooth muscle (Buus *et al.* 1998; Swärd *et al.* 2000), alternative possible pathways of rhoA/ROK-mediated Ca^{2+} sensitisation, such as alteration of actin filament dynamic equilibrium (Mehta & Gunst, 1999), require fuller investigation. For example, treatment with cytochalasin D, an inhibitor of F-/G-actin dynamic equilibrium, results in attenuation of intact uterine smooth muscle contractions in response to carbachol without altering $[Ca^{2+}]_i$ responsiveness (Taggart *et al.* 2001).

Agonist-stimulated cellular redistribution of rhoA and ROK

In resting uterine cells rhoA is diffusely distributed throughout the cytoplasm (Yu & López Bernal, 1998; Taggart *et al.* 1999) most probably existing in a rhoA–GDI (guanine nucleotide dissociation inhibitor) complex. Muscarinic stimulation of isolated uterine smooth muscle cells results in the translocation of rhoA from the cytoplasm to the cell periphery (Taggart *et al.* 1999). Membranous relocation of rhoA is essential for its Ca^{2+}-sensitising actions in a variety of smooth muscles (Gong *et al.* 1996; Fujihara *et al.* 1997). Notably, ROKα is similarly redistributed from the cytoplasm to the plasma membrane of intact uterine smooth muscle cells following identical conditions of receptor activation that result in *maintained* Ca^{2+}-sensitised contractions of permeabilised cells (Taggart *et al.* 1999). Ca^{2+} sensitisation thus occurs without significant relocalisation of ROK away from submembranous regions of the cell. The precise subcellular distribution of the trimeric MLCP, or the

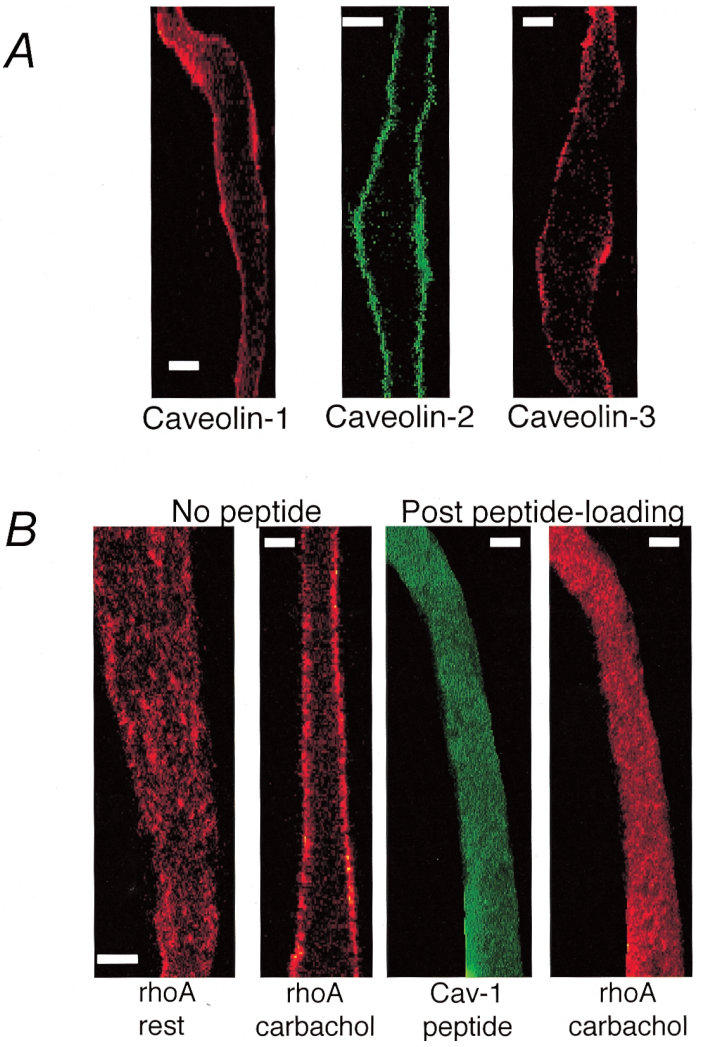

Figure 2

Caveolin and rhoA localisation in uterine smooth muscle. Laser scanning confocal microscopy of single uterine smooth muscle cells isolated from pregnant rat was used to establish the localisations of caveolins and rhoA. Central cell sections are illustrated. *A*, caveolin-1, -2 and -3 all exhibit a predominantly plasma membranous distribution in single myometrial cells. *B*, RhoA localisation before and after cellular introduction of the fluorescein isothiocyanate (FITC)-labelled (green) caveolin-1 scaffolding domain peptide. RhoA shows a stimulus-dependent relocalisation from the cytosol to the cell periphery in control cells. RhoA transocation is inhibited by the caveolin-1 scaffolding domain peptide. Scale bars represent 10 μm. Adapted from Taggart *et al.* (2000*b*).

targetting subunit of MLCP (MYPT) that is a major substrate for ROK, is unknown in smooth muscle. Although most MLCP activity is found in myofibrillar cell fractions (Alessi *et al.* 1992) substantial MYPT is present in membrane fractions (Kimura *et al.* 1996; Ito *et al.* 1997), has been shown to be regulated by interactions with acidic phospholipids (Ito *et al.* 1997) and, in confluent cultured cells, has been localised close to the plasma membrane (Hirano *et al.* 1999). On balance, it would appear, therefore, that the major subcellular site(s) of rhoA/ROK-mediated Ca^{2+} sensitisation in uterine smooth muscle lies in close proximity to the plasma membrane. Indeed, the ultrastructural arrangement of the smooth muscle cell – where contractile and cytoskeletal filaments exit membrane attachment sites at an acute angle to the cell axis – is consistent with a significant proportion of contractile load being sensed by the plasma membrane (Small & Gimona, 1998). In this manner it may be feasible for the effects of discrete changes in the activation state of myofilaments close to the plasma membrane to be transduced across the whole cell.

What might be the nature of the membrane structure(s) that co-ordinates the recruitment of downstream signalling molecules such as rhoA and ROK? Electron micrography of uterine smooth muscle illustrates that the membrane consists of periodic electron dense material – the dense plaques where actin filaments insert into the membrane – interspersed with rows of Ω-shaped invaginations known as caveolae (Taggart *et al.* 2000*b*). Caveolins are a family of proteins – three *main* mammalian isoforms exist (termed caveolin-1, -2 and -3) – critical to caveolar formation that, *in vitro*, interact with a variety of signal transducing molecules (reviewed by Okamoto *et al.* 1998 and Shaul & Anderson, 1998). A short cytoplasmic region of caveolin appears essential for the interaction with signal transduction molecules and has thus been termed the scaffolding domain. The possibility exists, therefore, that caveolae, as a result of the properties of caveolins, may be specialised plasmalemmal regions involved in the co-ordination of receptor-coupled signalling events.

Caveolins and uterine smooth muscle signal transduction

Use of isoform-specific antibodies indicates that each caveolin isoform is expressed in rat and human uterine smooth muscle irrespective of gestational status (Taggart *et al.* 2000*a*,*b*). Laser scanning confocal microscopy of single isolated rat uterine smooth muscle cells indicates that caveolins-1, -2 and -3 are all localised predominantly at the plasma membrane (Fig. 2). Increased intensity of staining is often observed at the cell extremities and membranous areas closely apposing the nucleus (Taggart *et al.* 2000*b*). Thus caveolin localisation is suitable for any potential interaction with signalling molecules redistributed to the plasma membrane following cell stimulation.

Introduction to single isolated uterine smooth muscle cells of the caveolin-1 scaffolding domain peptide completely inhibited rhoA translocation following muscarinic stimulation (Fig. 3). PKC_{α}, suggested to be important in agonist-induced Ca^{2+} sensitisation of other smooth muscles (Lee *et al.* 1999), also shows a receptor-coupled relocalisation to the plasma membrane of uterine smooth muscle cells (Taggart *et al.* 1999) that is inhibited by the caveolin-1 scaffolding domain peptide (Taggart *et al.* 2000*b*). Translocation was unaffected in sham-treated cells, or in cells treated with a scrambled version of the caveolin scaffolding domain peptide (Taggart *et al.* 2000*b*). These data suggest that both rhoA and PKC_{α} interact with caveolins via the N-terminal proximal scaffolding domain in intact uterine smooth muscle, results consistent with *in vitro* biochemical studies (Oka *et al.* 1997; Gingras *et al.* 1998). Furthermore, Couet *et al.* (1997) described caveolin binding motifs typical of many signalling molecules found to interact with caveolin and both PKC_{α} and rhoA contain corresponding sequences. In particular, the sites of monoglucosylation (T^{37}) and ADP-

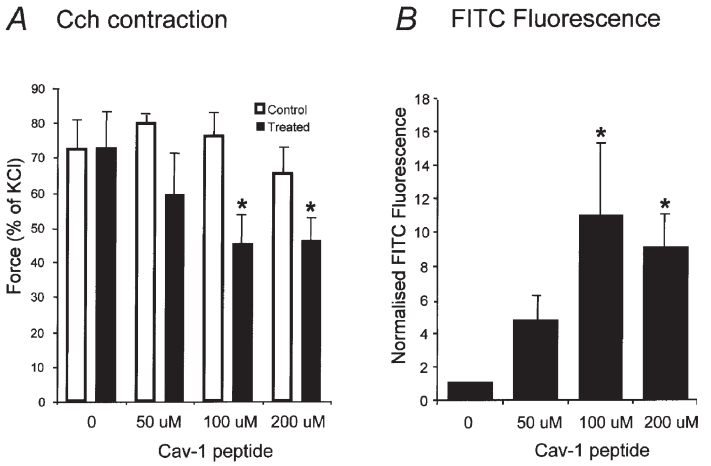

Figure 3

Inhibition of uterine smooth muscle contraction by caveolin-1 scaffolding domain. The FITC-labelled caveolin-1 scaffolding domain peptide was chemically loaded into intact uterine smooth muscle strips by a procedure similar to that described by Taggart *et al.* (2000*b*). Contractions to CCh (100 μM) in 0 Ca^{2+} solution were dose-dependently inhibited by the scaffolding domain (*A*) in parallel with increased fluorescent labelling of the tissues (*B*). Contractions were unaffected in sham-treated tissues.

ribosylation (N^{41}) on rhoA are both contained within the putative caveolin binding motif (^{34}YVPTVFENY^{41}); this motif also overlaps with a region important for determining rhoA binding with ROK (Fujisawa *et al.* 1998). It is presently unknown if caveolin directly interacts with ROK but it too contains putative caveolin binding motifs in the catalytic domain of the molecule (^{151}WVVQLFCAF^{159} and ^{164}YLYMVMEY^{171}). Collectively, these data suggest that caveolin interacts with, and regulates activity of, rhoA (and possibly ROK). Consistent with such a mechanism of action is the observation that introduction of the caveolin-1 scaffolding domain peptide to intact uterine smooth muscle resulted in inhibition of contractile function: contractions in response to carbachol (but not those in response to KCl) were significantly reduced whilst responses in sham-treated tissues remained unaltered (Fig. 3).

Conclusions

Receptor-stimulated membranous recruitment of key signalling molecules rhoA and ROK appears to be crucial for agonist-induced Ca^{2+} sensitisation in single uterine smooth muscle cells. Caveolae, as a result of the regulatory actions of caveolins, are suggested to be specialised plasmalemmal regions involved in this integration of extracellular signals and intracellular effectors. Such discrete membranous localisation of signal transducing events is likely to contribute to the efficacy of receptor-coupled contractile activation of uterine smooth muscle.

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