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Spontaneous localised rises of intracellular Ca^{2+} in detrusor smooth muscle

C. Wu, G. Sui and C. Fry

Institute of Urology and Department of Medicine, University College London, London, UK

Increased spontaneous contractile activity in detrusor smooth muscle may contribute to unstable bladder contractions and increased bladder tone associated with urinary incontinence (1). The underlying mechanisms for enhanced contractile function are not clear, but an altered intracellular Ca^{2+} regulation is important (2, 3). The present investigation characterised localised rises of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, in detrusor myocytes from guinea-pig and human bladders and examined their role in generating spontaneous activity.

Guinea-pigs were humanely killed. Human detrusor samples were obtained from surgical operations, with Ethical Committee approval and patient consent. Single detrusor myocytes were dissociated from muscle strips using a collagenase-based enzyme mixture (4). Cells were superfused with a Tyrode's solution, gassed with 95% O_2/5% CO_2, at 37°C, pH7.4. Cells were loaded with Fluo-4 AM (1μM) and $[Ca^{2+}]_i$ was measured as fluorescence intensity emitted at 515-530 nm, excited at 488 nm, scanned with an argon laser, using a BioRad Radiance 2100 system. Images were obtained either by line scan or x, y plane scan and processed with LaserPix program. Parameters for quantifying Ca^{2+} sparks were amplitude (F/F_0), full duration half-maximum (FDHM) and full-width half-maximum (FWHM). Data are expressed as mean±SD and Student's t-test was used ($p<0.05$)

Localised rises of $[Ca^{2+}]_i$, in the form of discrete, fast sparks, were observed both in un-stimulated guinea-pig (F/F_0 1.52±0.33, FDHM 160±89ms, FWHM 2.08±0.85μm, frequency 0.38±0.27Hz, n=89) and human detrusor myocytes (F/F_0 1.45±0.37, FDHM 142±74ms, FWHM 2.61±0.96μm, frequency 0.27±0.16Hz, n=72). Larger cluster-like local activities also occurred. There was generally one spark-generating site inside the cell, but multiple release sites could also be observed. These localised sparks either remained local or spread to nearby areas. Repetitive firing of Ca^{2+} sparks and their fusion were able to progress into Ca^{2+} waves, leading to synchronised whole cell $[Ca^{2+}]_i$ transients. The spark activities could be abolished by 10-20μM ryanodine (n=10), as well as 20μM cyclopiazonic acid (n=6) and 1μM thapsigargin (n=5). A low caffeine concentration (1mM) and a moderate rise of extracellular KCl (to 20mM) increased spark frequencies (caffeine: 162±14% of control, $p<0.01$ n=4; KCl: 140±25% of control, $p<0.05$, n=3) and led to subsequent whole cell transients.

These observations show that localised Ca^{2+} rises can occur spontaneously in detrusor smooth muscle cells at rest. The temporal and spatial summation of these local Ca^{2+} oscillating events leads to spontaneous global activity in detrusor muscle. These local transients are controlled by the activity of ryanodine receptors, and can be up-regulated by membrane depolarisation.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Synchronization of Ca^{2+} oscillations through voltage-dependent modulation of store Ca^{2+} release

M.S. Imtiaz[1], C. Katnik[2], D.W. Smith[3] and D.F. van Helden[1]

[1]The Neuroscience Group, The School of Biomedical Sciences, The University of Newcastle, Callaghan, NSW, Australia, [2]Pharmacology and Therapeutics, University of Southern Florida, Tampa, FL, USA and [3]Department of Civil and Environmental Engineering, The University of Melbourne, Melbourne, VIC, Australia

The mechanical activity of many smooth muscles is controlled by a cyclical depolarization known as slow waves (Bolton, 1971). Slow waves result through rhythmic Ca^{2+} release from intracellular Ca^{2+} stores through inositol 1,4,5-trisphosphate (IP_3) sensitive receptors and Ca^{2+}-induced Ca^{2+} release. Ca^{2+} oscillations are transformed into membrane depolarizations by generation of a Ca^{2+}-activated inward current. Importantly, the store Ca^{2+} oscillations that underlie slow waves are entrained across many cells over large distances (van Helden & Imtiaz, 2003). It has been shown that IP_3 receptor-mediated Ca^{2+} release is enhanced by membrane depolarization (Suzuki & Hirst, 1999; van Helden et al. 2000), and it is this positive feedback that underlies the long-range entrainment of Ca^{2+} stores. The present study examines the mechanisms underlying such store entrainment.

In gap junction connected cells diffusion of Ca^{2+} or IP_3 across gap junctions have been implicated in synchronization of Ca^{2+} oscillations. In the present study we investigate Ca^{2+} store entrainment through depolarization-induced IP_3 receptor-mediated Ca^{2+} release. This mechanism is significantly different from the chemical coupling-based class of models as membrane potential has a coupling effect over distances several orders of magnitude greater than either diffusion of Ca^{2+} or IP_3 through gap junctions.

Experimental observations predicate that electrically coupled cells can interact and modulate Ca^{2+} excitability and oscillations of other cells through voltage dependent enhancement of store Ca^{2+} release. We encapsulate this in a model where; 1) each local oscillator is composed of a cytosolic-store Ca^{2+} excitable system, 2) local Ca^{2+} oscillations are coupled to membrane potential, and, 3) membrane potential influences the local Ca^{2+} oscillator through a positive feedback loop. We construct a coupled cell pair according to the schema outlined above using our previously presented single cell model (Imtiaz et al. 2002). Here we use voltage-dependent IP_3 synthesis to model the positive feedback of membrane potential on cytosolic-store excitability.

The effect of electrical coupling strength on synchronization of a cell pair is studied. It is shown that weak electrical coupling is sufficient to synchronize even heterogeneous cell pairs. A comparison is made between electrical and chemical coupling (through diffusion of Ca^{2+} or IP_3). It is shown that chemical coupling is not effective when cells are weakly coupled and have different intrinsic frequencies. The result of this study show that

electrical coupling acting through voltage-dependent modulation of store Ca^{2+} release is able to synchronize oscillations of cells even when cells are weakly coupled (or widely separated) and/or have different intrinsic frequencies of oscillation.

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Mitochondrial and cytosolic calcium transients in adult cardiomyocytes detected using targeted aequorin

C.J. Bell, G.A. Rutter and E.J. Griffiths

Bristol Heart Institute and Dept of Biochemistry, University of Bristol, Bristol, UK

Intramitochondrial free calcium ($[Ca^{2+}]_m$) is an important regulator of ATP production in the heart, and may also modulate whole-cell Ca^{2+} signalling. However, research has been hampered by the difficulty of measuring $[Ca^{2+}]_m$ in living myocytes. Here, we used an adenovirus containing aequorin targeted to either cytosol or mitochondria to determine whether beat-to-beat oscillations in $[Ca^{2+}]_m$ could be detected in adult myocytes.

Adult rats were humanely killed and ventricular myocytes isolated by collagenase digestion. Myocytes were then cultured in the presence of adenovirus containing aequorin targeted to either mitochondria or cytosol. $[Ca^{2+}]$ was measured in cells electrically stimulated at 2Hz by collecting aequorin light output using a photon counting camera (10-50 cells per field). Results are presented as means ± S.E.M.; n values refer to the number of separate fields of cells studied. Correct localisation of the aequorin constructs to the mitochondria and cytosol was confirmed by immunocytochemistry using antibodies against the Haemagglutinin tag contained in the constructs. The images obtained were indicative of correct compartmentalisation of the expressed proteins.

Ca^{2+} transients were clearly visible in both cytosol and mitochondria. Although resting (diastolic) values could not be accurately estimated due to the high signal to noise ratio at low $[Ca^{2+}]$, systolic values were quantifiable. In the presence of the β adrenergic agonist isoproterenol systolic $[Ca^{2+}]_c$ increased from 0.9 μM ± 0.04 (n=36) to 1.65 μM ± 0.07 (n=19) and $[Ca^{2+}]_m$ from 0.9 μM ± 0.09 (n=11) to 1.2 μM ± 0.11 (n=10). We also performed these experiments in the presence of the mitochondrial Na^+-Ca^{2+} exchange inhibitor clonazepam. Addition of 50 μM clonazepam caused a transition from beat-to-beat transients to a sustained $[Ca^{2+}]$ of 1.2 μM ± 0.08 (n=16) in mitochondria (in the presence of isoproterenol). Conversely, clonazepam reduced the amplitude of cytosolic transients from 1.0 μM ± 0.02 (n=10) to 0.79 μM ± 0.04 (n=10) and from 1.91 μM ± 0.09 (n=8) to 1.48 μM ± 0.09 (n=10) in the presence of isoproterenol.

Since changes in $[Ca^{2+}]_m$ are known to regulate mitochondrial energy production, mitochondrial [ATP] was measured using the expression of targeted luciferase. No beat-to-beat changes in $[ATP]_m$ could be observed using this method, although a sustained rise in $[ATP]_m$ was observed over longer time periods when myocytes were stimulated from rest in the presence of isoproterenol.

These results implicate the mitochondrial efflux pathway in modulation of cytosolic Ca^{2+} transients. The lack of change in mitochondrial ATP in beating myocytes indicates that ATP supply and demand are normally well matched. However, $[ATP]_m$ can increase upon rapid stimulation of resting cells.

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Spontaneous electrical activity in the guinea-pig prostate gland

D. Nguyen[1], B. Exintaris[1] and R. Lang[2]

[1]Pharmaceutical Biology and Pharmacology, Monash University, Parkville, VIC, Australia and [2]Physiology, Monash University, Clayton, VIC, Australia

We have previously shown that a layer of c-Kit-immunoreactive interstitial cells lie between the smooth muscle and epithelial layers of the individual acini in the guinea-pig prostate gland (1). These interstitial cells displayed many of the distinguishing features of interstitial cells of Cajal (ICC), the pacemaker cells of the gastrointestinal tract. In this study, we have recorded at least 2 distinct types of spontaneous electrical activity in the stroma of the guinea pig prostate using standard intracellular microelectrode-recording techniques. Prostate glands were removed from humanely killed guinea-pigs (250-350g). Saccular glands were pinned to the bottom of an organ bath, which was subsequently mounted on an inverted microscope stage and perfused with physiological saline solution. Slow waves consisted of a depolarizing transient (14 mV in amplitude) with 1-6 spikes superimposed, pacemaker potentials occurred at the same frequency as slow waves, but were larger in amplitude (40mV), and spontaneous transient depolarizations (STD) occurred irregularly in the presence of the slow wave or pacemaker activity. In the presence of the L-type Ca2+ channel blocker, nifedipine (1μM), the spike potentials superimposed on the depolarising transient of control slow waves were abolished. Other slow wave parameters were not affected (n=18). In contrast, nifedipine had no significant effects on any of the measured parameters of the pacemaker potentials recorded in 5 experiments. In the presence of nifedipine, cyclopiazonic acid (10μM)(n=6) which depletes internal Ca2+ stores by blocking the Ca-ATPase (SERCA) pump on the sarcoplasmic reticulum or the mitochondrial inhibitors, FCCP (10-10μM)(n=4), CCCP (1-10μM)(n=4) or rotenone (1-10μM)(n=3) abolished electrical activity. These results demonstrate that spontaneous electrical activity in the guinea-pig prostate gland is dependent on the release and/or uptake of Ca2+ from intracellular Ca2+ stores and mitochondria. Finally, we speculate that the depolarizing component of slow wave represents the propagated responses of pacemaker potentials arising from our previously identified c-Kit immunoreactive interstitial cells.

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Tumour necrosis factor-α-induced RhoA activation in airway smooth muscle cells: Potential involvement of lipid raft domains

G.F. Nixon and I. Hunter

School of Medical Sciences, University of Aberdeen, Aberdeen, UK

The inflammatory cytokine, tumour necrosis factor-α (TNF), may be a key component of airway hyper-responsiveness producing an increased contractility. Our previous study has demonstrated that this increased contractility is the result of TNF-induced RhoA activation leading to an increased sensitivity to Ca^{2+} (Hunter et al, 2003). The aim of this study was to determine the intracellular mechanisms that result in TNF-induced RhoA activation in primary cultured human airway smooth muscle (ASM) cells (commercially obtained). RhoA activation was assayed using the GST-rhotekin "pull-down" protocol (Hunter et al, 2003). Pretreatment of cells with 10 mM methyl β-cyclodextrin (Mβ-CD) (which depletes cholesterol thereby disrupting lipid raft domains) for 30 minutes resulted in a significant decrease in TNF-induced RhoA activation at 1 minute and 5 minutes stimulation (Mβ-CD treated 1.1 ± 0.2 fold increase, n=5; control 4.2 ± 0.4 fold increase, n=5, mean ± s.e.m., student's t-test $p<0.05$). To determine if TNF receptors are present in lipid rafts, biotin-coupled TNF was used to label receptors, followed by incubation with a streptavidin-coupled fluorophore. TNF receptors were observed to co-localize predominantly with lipid raft regions of the plasma membrane in airway smooth muscle cells. Sucrose gradient fractionation of ASM cell extracts was carried out to separate Triton X-100 soluble and insoluble (lipid raft) fractions. Insoluble fractions, immunoblotted for proteins potentially involved in TNF-induced activation of RhoA, contained TNF receptor 1, several TNF receptor accessory proteins as well as RhoA. Caveolin-1, a marker of lipid rafts, was present only in raft fractions. Recruitment of TNF to lipid rafts following stimulation may be an important part of this signalling process. To assess if this occurs, cells were incubated with TNF and cell extracts separated into Triton X-100 soluble and insoluble (lipid raft) components. There was a significant increase in TNF receptor 1 recruitment to insoluble fractions following TNF stimulation for 1 minute (3.2 ± 0.4 fold increase compared to control, n=3). This was maintained after 5 minutes TNF stimulation. In conclusion, TNF-induced RhoA activation in human airway smooth muscle cells is dependent on functional lipid raft domains that contain components of TNF signalling, as well as RhoA. Following TNF stimulation, TNF receptors are recruited to the lipid rafts regions of the plasma membrane. Lipid rafts may therefore form an important platform for TNF-induced RhoA activation in ASM cells.

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Microdomains and Vascular Smooth Muscle Function

P. Hellstrand, K. Sward and S. Albinsson

Dept Exp. Med. Sci., Lund University, Lund, Sweden

Smooth muscle is adapted for contraction but also shows a high degree of plasticity of differentiation and growth in response to environmental influences. Signals carried by a limited number of intracellular second messengers need to be integrated in space and time to fulfill a plethora of specific tasks, and increasing evidence indicates that this is achieved partly by an intricate arrangement of local signal systems organized in microdomains. An example of this is the clustering of receptors and ion channels in cholesterol-rich membrane regions (caveolae or lipid rafts). Part of the contractile response to endothelin-1 (ET-1) in rat tail artery is due to activation of Ca^{2+} inflow via TRPC1 channels, which is sensitive to extraction of membrane cholesterol, correlating with disruption of caveolae (Bergdahl *et al.* 2003). This suggests clustering of signal molecules into functional units in caveolae. Coupling of caveolae with growth signals is suggested by studies in the isolated rat portal vein, showing that stretch of the vessel wall increases tyrosine phosphorylation of several proteins in a membrane fraction containing the caveolar marker protein caveolin-1 (Zeidan *et al.* 2003). Stretch also causes activation of ERK1/2 and increased protein synthesis in the portal vein, but these effects were abolished after depletion of cholesterol. Part of the trophic response to stretch may be mediated by endogenous production of angiotensin II and ET-1, and it is interesting that ERK phosphorylation in response to ET-1 but not angiotensin II was found to be inhibited by cholesterol depletion. This demonstrates that local signal pathways responding to the same stimulus (stretch) and mediating the same downstream effect (ERK activation) may differ in their relationship to caveole.

The differentiated smooth muscle phenotype is marked by a limited number of contractile and cytoskeletal proteins, whose expression is regulated by serum response factor (SRF; reviewed by Owens *et al.* 2004). One factor shown to be important for SRF-dependent smooth muscle specific gene expression is the level of actin polymerization, which may influence the translocation of SRF and/or coactivators to the nucleus. In the portal vein, mechanical strain increases actin polymerization by activating its Rho-dependent regulatory pathway, and concomitantly stimulates the synthesis of differentiation marker proteins (Albinsson *et al.* 2004). Thus stretch of the intact vascular wall activates both an ERK-dependent increase in protein synthesis and a specific Rho-dependent differentiation pattern. At the membrane level, stretch causes biphasic activation of focal adhesion kinase (FAK), where an early peak (15 min) correlates in time with stretch-induced ERK phosphorylation, while a later sustained increase (24 h) correlates with Rho activation. Therefore, the domain organization of signal molecules into caveolae and focal adhesions in the plasma membrane may be essential for transduction of signals regulating contractile activity, growth, and cellular phenotype. Further elucidation of these mechanisms will clarify how environmental conditions influence smooth muscle function, of profound importance for pathophysiological adaptation.

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