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The sources and sequestration of Ca^{2+} contributing to neuroeffector Ca^{2+} transients in the mouse vas deferens

Keith L. Brain, Alina M. Cuprian, Damian J. Williams and Thomas C. Cunnane

University Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK

The detection of focal Ca^{2+} transients (called neuroeffector Ca^{2+} transients, or NCTs) in smooth muscle of the mouse isolated vas deferens has been used to detect the packeted release of ATP from nerve terminal varicosities acting at postjunctional P2X receptors. The present study investigates the sources and sequestration of Ca^{2+} in NCTs. Smooth muscle cells in whole mouse deferens were loaded with the Ca^{2+} indicator Oregon Green 488 BAPTA-1 AM and viewed with a confocal microscope. Ryanodine (10 μM) decreased the amplitude of NCTs by 45 ± 6 %. Cyclopiazonic acid slowed the recovery of NCTs (from a time course of 200 ± 10 ms to 800 ± 100 ms). Caffeine (3 mM) induced spontaneous focal smooth muscle Ca^{2+} transients (sparks). Neither of the T-type Ca^{2+} channel blockers $NiCl_2$ (50 μM) or mibefradil dihydrochloride (10 μM) affected the amplitude of excitatory junction potentials (2 ± 5 % and −3 ± 10 %) or NCTs (−20 ± 36 % and 3 ± 13 %). In about 20 % of cells, NCTs were associated with a local, subcellular twitch that remained in the presence of the α_1-adrenoceptor antagonist prazosin (100 nM), showing that NCTs can initiate local contractions. Slow (5.8 ± 0.4 μm s^{-1}), spontaneous smooth muscle Ca^{2+} waves were occasionally observed. Thus, Ca^{2+} stores initially amplify and then sequester the Ca^{2+} that enters through P2X receptors and there is no amplification by local voltage-gated Ca^{2+} channels.

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Corresponding author K. L. Brain: University Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK. Email: keith.brain@pharm.ox.ac.uk

The neurotransmitter ATP initiates excitatory junction potentials (EJPs) and a fast component of contraction by acting at $P2X_1$ receptors on smooth muscle cells of the rodent vas deferens (Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Morris & Gibbins, 1992; Mulryan *et al.* 2000). Recently, we reported that nerve stimulation intermittently initiates focal increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in those parts of smooth muscle cells that are closely apposed to nerve terminal varicosities, even though there is no intermittence of the action potential in the terminal; these focal Ca^{2+} transients have been termed NCTs (Brain *et al.* 2002). NCTs are abolished at most junctions by α,β-methylene ATP, which desensitises $P2X_1$ receptors, suggesting that they are initiated by the activation of P2X receptors (Brain *et al.* 2002). NCTs are of interest as a tool for monitoring neurotransmitter release, on an impulse-to-impulse basis, simultaneously from a large number of varicosities including sequential varicosities on the same nerve terminal branch. The existence of such focal Ca^{2+} transients may have important implications for the contraction and subcellular regulation of smooth muscle cells.

While P2X receptor activation is obligatory for NCT initiation, the source of Ca^{2+} and the mechanisms terminating these Ca^{2+} transients have not yet been determined. The most obvious source of Ca^{2+} following the activation of P2X receptors is the influx of Ca^{2+} through the P2X receptor itself, which is known to have a high Ca^{2+} permeability in smooth muscle cells (Benham & Tsien, 1987). However, it is also possible that local Na^+ influx following P2X receptor activation could cause sufficient local depolarisation to open either high-voltage activated (L-type) or low-voltage activated (T-type) Ca^{2+} channels. It has previously been shown that L-type Ca^{2+} channels do not contribute to NCTs (Brain *et al.* 2002), but there has been no previous investigation of T-type channels.

Intracellular Ca^{2+} stores have, for some time, been implicated in the intermittent, subcellular increases in Ca^{2+} concentration known as sparks or quarks in excitable cells. Such events were first studied in cardiac and skeletal muscle cells, but they also occur in many smooth muscle cells (for reviews, see Jaggar *et al.* 2000; Pabelick *et al.* 2001), including myocytes isolated from the guinea-pig vas deferens (Ohi *et al.* 2001; White & McGeown, 2003).

In memory of Dr S. J. Trout.

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Any small local Ca^{2+} transient, such as that following the influx of Ca^{2+} through $P2X_1$ receptors, could potentially trigger Ca^{2+}-induced Ca^{2+}-release (CICR). The present work explores the role of intracellular Ca^{2+} stores and T-type voltage-gated Ca^{2+} channels in the genesis and termination of NCTs.

METHODS

Ca^{2+} indicator loading

Eight- to twelve-week-old Balb/c mice (Harlan, UK) were killed by cervical fracture and both vasa deferentia removed. Efforts were made to minimise the number of animals used; all experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. The connective tissue around each vas deferens was carefully dissected in order to obtain clear images of smooth muscle cells and to remove any ganglia or isolated nerve cell bodies located close to the vas deferens. Each vas deferens was then exposed to 10 μM Oregon Green 488 BAPTA-1 AM (Oregon-BAPTA; Molecular Probes) in 1 % DMSO/0.2 % pluronic F-127 in physiological salt solution (PSS) for 2 h at 36 °C. Each vas deferens was then rinsed in bubbled PSS for at least 10 min prior to transfer to an organ bath mounted on the stage of a confocal microscope. The PSS contained (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 1.3 and glucose 11.1. The pH was maintained at 7.4, and the solution oxygenated, by continuously bubbling with 95 % O_2/5 % CO_2.

Confocal microscopy

The vas deferens was placed in a chamber that was continuously perfused with the standard PSS (bath temperature 31–32 °C). The base of the chamber was a coverslip; images were acquired with a Leica NT inverted confocal microscope. The effective confocal pinhole was set to 3.65 Airy discs; this gives a large depth of field. Field stimuli (pulse width 0.6 ms; amplitude 10 V) were applied with parallel Ag/AgCl electrodes located near the prostatic end of the aganglionic vas deferens; stimuli were synchronised with the start of image acquisition, so that the time between each stimulus and recording was fixed. Unless stated otherwise, images were captured at twice the stimulus frequency (1–2 Hz), so that a stimulus occurred on every second frame. This protocol provided a control image prior to every stimulus and hence allowed consecutive $[Ca^{2+}]$ transients from the same location to be distinguished. Confocal images were continuously acquired for 30 s to generate one image set. Such sets were acquired every 2 min. Eight such sets were acquired for each preparation in order to measure the control response at each neuroeffector junction. Preparations were then exposed to drugs for either 90 min (ryanodine) or 20 min (all other drugs) to ensure adequate equilibration, and then a further eight sets of images were acquired. During incubation in ryanodine (and in the corresponding control), field stimuli of 10 impulses at 10 Hz were applied every 30 s because the action of ryanodine is use dependent (see, for example, Kano *et al.* 1995; Smith & Cunnane, 1996).

Image analysis

Image analysis was performed with NIH Image version 1.62 (from http://rsb.info.nih.gov/nih-image/) or Image SXM version 1.68 (from http://reg.ssci.liv.ac.uk). Adjacent cells were distinguished from one another by step-like changes in the fluorescent signal at cell boundaries, as even adjacent cells were differentially loaded with Ca^{2+} indicator. In some cases the raw images were too dim to confidently distinguish cell boundaries. In such cases an average from 100 consecutive frames was calculated and this averaged image (with its greater signal to noise ratio) was used to count cells. Local, discrete changes in $[Ca^{2+}]_i$ were detected using a modification of previously-described custom-written macros (Brain *et al.* 2002). Raw images were convolved with a Gaussian filter of half-width 1.5 μm. The ratio of the fluorescent intensities on consecutive images was calculated on a pixel-by-pixel basis. The Particle Analysis algorithm of NIH Image was used to detect focal changes in the ratio of fluorescent intensities that were greater than one S.D. above the noise in the control images over a continuous area of at least 2.5 μm^2. A threshold was set, equal to the mean background signal of the image, below which the algorithm did not search for events.

When measuring the kinetics of NCTs, the effects of movement were reduced with the 'Auto Register' facility of Image SXM (i.e. the use of a cross-correlation algorithm, based on the fast Fourier transform of each image, to align all images in a set). The change in fluorescent signal was measured within a rectangular region (area 20–60 μm^2) that completely enclosed the $[Ca^{2+}]_i$ transient. The measurement box was larger than the area of the $[Ca^{2+}]_i$ transient so that the entire change in fluorescent signal was measured. The time course of recovery (the time taken to fall to e^{-1}) of focal $[Ca^{2+}]_i$ transients was calculated using an exponential curve fit to the average response (over the first 1 s after the point of first detection) at a given junction. Least squares curve fitting was performed with either Excel (Microsoft) or GraphPad Prism 3.0a (GraphPad Software Inc.).

Electrophysiology

Conventional intracellular recording techniques were used to record excitatory junction potentials (EJPs) in smooth muscle cells (see Brock & Cunnane, 1992). Each vas deferens was superfused with PSS and drugs were applied by swapping the perfusion solution to one containing the drug at the required final bath concentration. Stimuli (rectangular pulses, 0.1 ms, amplitude 10 V) were delivered through Ag/AgCl electrodes positioned around the proximal end of the vas deferens. Microelectrodes were filled with 5 M potassium acetate and had tip resistances of 40–80 MΩ. The membrane potential was measured with an Axoclamp 2A (Axon Instruments, California, USA) in bridge mode, then digitised with a PowerLab system and Chart 4.2 (both from ADInstruments, Chalgrove, UK). The time course of repolarisation (decay time constant) was the time taken to fall from 0.90 to 0.33 of the peak amplitude (i.e. the time taken to fall by e^{-1}).

Drug sources and preparation

Cyclopiazonic acid (CPA; Tocris, Avonmouth, UK) and ryanodine (Sigma-Aldrich, Poole, Dorset, UK) were stored frozen (−20 °C) in aliquots at a concentration of 10 mM in DMSO; $NiCl_2$ was stored frozen at a concentration of 1 M in distilled water. Caffeine (Sigma-Aldrich, Dorset, England) solutions were prepared on the day of the experiment at the working concentration. Mibefradil dihydrochloride (donated by Dr E. V. Gutknecht and P. Weber, Hoffmann-La Roche, Basel, Switzerland) stock solutions had a concentration of 10 mM in distilled water; aliquots were frozen until required.

When comparing the amplitudes of NCTs it was noted that the amplitude distribution is skewed and so a non-parametric test (Wilcoxon matched pairs test) was applied. All other statistical

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tests were two-tailed Student's *t* tests; the results are reported as significant when $P < 0.05$. In the confocal imaging experiments, the assumption was made that each junction behaves independently and hence the *n*-number for the statistical test is the number of junctions studied (n_j).

RESULTS

In each vas deferens the Ca^{2+} indicator Oregon-BAPTA filled the cytoplasm of smooth muscle cells within one to two cell layers of the surface. Field stimulation (1 Hz) of vasa deferentia induced highly intermittent, focal $[Ca^{2+}]_i$ transients in Oregon-BAPTA-filled smooth muscle cells (Fig. 1*A*). These events occurred in characteristic locations (or clusters) within the cells (Fig. 1*B*) at sites attributed to single varicosity – smooth muscle cell neuroeffector junctions; these focal $[Ca^{2+}]_i$ transients are referred to as NCTs (Brain *et al.* 2002). Within each cluster, the mean probability that a stimulus evoked an NCT was 0.020 ± 0.002 ($n_j = 53$ junctions; number of smooth muscle cells (n_{sm}) = 10; number of preparations (n_p) = 5; mean ± S.E.M.). When α,β-methylene ATP (10 μM), which desensitises $P2X_1$ receptors, was bath applied for 30 min, NCTs were abolished at the majority of junctions (64/68). At the remaining junctions (4/68) the amplitude of the evoked Ca^{2+} transient was reduced (by >40 % at each junction), and the probability of detecting an NCT was reduced by 60 ± 20 %.

About 20 % of smooth muscle cells (7 of 35 cells) contained at least one junction where NCTs were immediately followed by a local twitch (commencing within 0.5 s of the NCT). In such cases the smooth muscle cell deformed towards the site of the NCT (see the supplementary material), although not every NCT in these cells induced a contraction. It appeared, subjectively, that a contraction was more likely when the local Ca^{2+} transient was of greatest amplitude. No distinguishing feature was identified that could predict which cells would locally contract. In all three preparations in which the α_1-adrenoceptor antagonist prazosin (100 nM) was applied, the local contractions associated with NCTs remained after at least 20 min of exposure.

Ca^{2+}-induced Ca^{2+} release

While the activation of P2X receptors is necessary for the generation of NCTs, the potential role of intracellular Ca^{2+} stores in amplifying the local $[Ca^{2+}]_i$ change, or in sequestering the remaining Ca^{2+}, is unknown. After incubating vasa deferentia in ryanodine (10 μM) for 90 min, the amplitude of NCTs (during field stimulation at a frequency of 2 Hz) was reduced by 45 ± 6 % ($n_j = 42$; $n_p = 7$; Fig. 2). In the continued presence of ryanodine, the application of caffeine (3 mM) did not elicit a detectable change in the smooth muscle Ca^{2+} concentration ($n_p = 3$ of 3). In control preparations (when only the vehicle was present for 90 min) there was no significant change in the amplitude of NCTs (+9 ± 10 %; $n_j = 13$; $n_p = 3$).

During field stimulation (2 Hz), the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA) had no significant effect on the amplitude of NCTs (3 ± 12 %; $n_j = 12$; $n_{sm} = 4$; $n_p = 3$; Fig. 3). The rate of recovery of the Ca^{2+} concentration slowed upon exposure

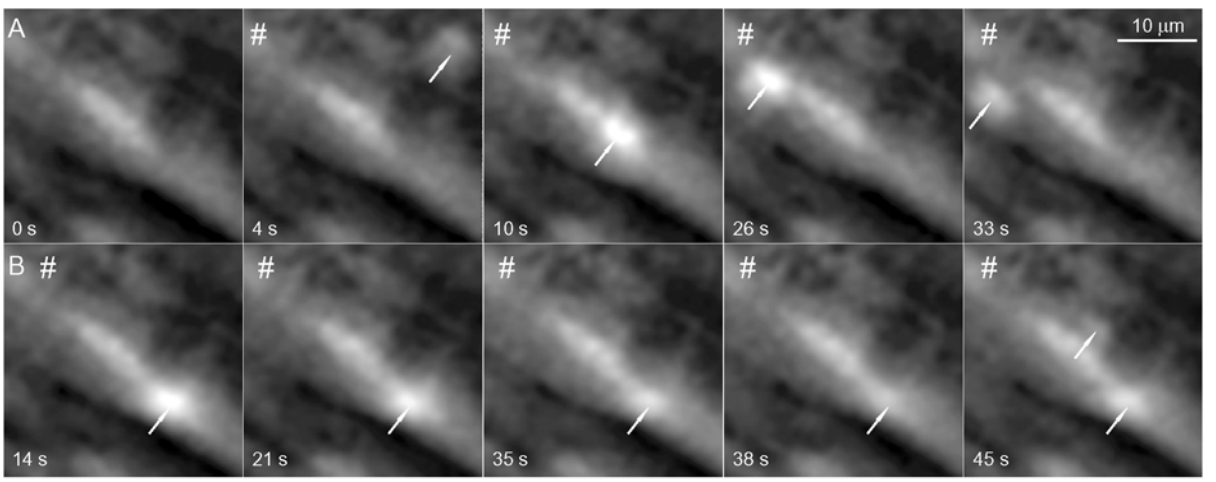

Figure 1. Purinergic neuroeffector Ca^{2+} transients (NCTs)

A small region close to the surface of the mouse vas deferens is shown during field stimulation (#) at 1 Hz (while recording images at 2 frames s^{-1}). Smooth muscle cells are heterogeneously loaded with the Ca^{2+} indicator Oregon-BAPTA. *A*, a set of selected frames to demonstrate that NCTs (arrows) are intermittently evoked at several sites within smooth muscle cells. The time at which the image was acquired is marked on each frame. *B*, another selection of images, from the same recording, showing all NCTs arising at a given site. Each frame should be compared with the control frame (*A*, frame 1). *B*, frame 5, shows synchronous events at two sites within the same smooth muscle cell.

to CPA, from a time constant of 200 ± 10 ms to 800 ± 100 ms (n_j = 12; n_{sm} = 4; n_p = 3).

Thapsigargin (1 μM), another sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, also had no detectable effect on the amplitude of NCTs (−20 ± 10 %; n_j = 9; n_{sm} = 5; n_p = 4), and slowed the time course of recovery (by 12 ± 6 %; n_j = 9; n_{sm} = 5; n_p = 4).

When exposed to 3 mM caffeine there was a 16-fold increase in the frequency of spontaneous focal $[Ca^{2+}]_i$ transients (Fig. 4; n_{sm} = 5; n_p = 3), which were not necessarily grouped in NCT clusters (Fig. 4).

T-Type Ca^{2+} channels

In some smooth muscle cells, T-type Ca^{2+} channels contribute to the EJP and the opening of these channels may produce local changes in $[Ca^{2+}]_i$. However, during stimulation at 0.33 Hz, the T-type Ca^{2+} channel blocker Ni^{2+} (at 30 μM, 50 μM or 300 μM) did not significantly change the amplitude of EJPs (5 ± 7 %; 4 ± 3 %; 7 ± 30 %, respectively; n_p = 4) or the rate of repolarisation (0 ± 7 %; 9 ± 14 %; 8 ± 12 %, respectively; n_p = 4). At a high concentration (300 μM), Ni^{2+} shifted the resting membrane potential towards more positive values (from −4 ± 3 mV to −69 ± 2 mV; n_p = 4; $P \leqslant 0.05$; Fig. 5*A*). Another T-type Ca^{2+} channel blocker (mibefradil; 10 μM) had no significant effect on the amplitude of EJPs (−3 ± 10 %, n_p = 6; Fig. 5*B*), but did slightly slow the rate of repolarisation of the EJP (by 14 ± 6 %). At a higher concentration (20 μM), mibefradil reduced the amplitude of excitatory junction potentials (by 37 ± 10 %; n_p = 6), slowed the rate of repolarisation (by 44 ± 16 %) and caused a significant membrane potential depolarisation (from −83 ± 1 mV to −71 ± 5 mV). Given that the higher concentrations of Ni^{2+} (300 μM) or mibefradil (20 μM) caused effects that could not readily be attributed to T-type Ca^{2+} channel inhibition, lower concentrations were used in confocal imaging experiments.

Another measure of neurotransmitter release is the probability of detecting an NCT at a given junction following a stimulus (P_{NCT}). Neither Ni^{2+} (100 μM) nor mibefradil (10 μM) had any significant effect on P_{NCT} (for Ni^{2+} experiments, control, 0.018 ± 0.004; Ni^{2+}, 0.021 ± 0.005; n_j = 18; n_{sm} = 6; n_p = 3; for mibefradil experiments, control, 0.014 ± 0.003; mibefradil, 0.017 ± 0.003; n_j = 35; n_{sm} = 5; n_p = 3).

It is possible that Na^+ influx through $P2X_1$ receptors could activate local voltage-gated Ca^{2+} channels. However, neither Ni^{2+} (100 μM; Fig. 5*C*) nor mibefradil (10 μM; Fig. 5*D*) affected the amplitude of NCTs (for Ni^{2+}: −20 ± 36 %; for mibefradil: 3 ± 13 %).

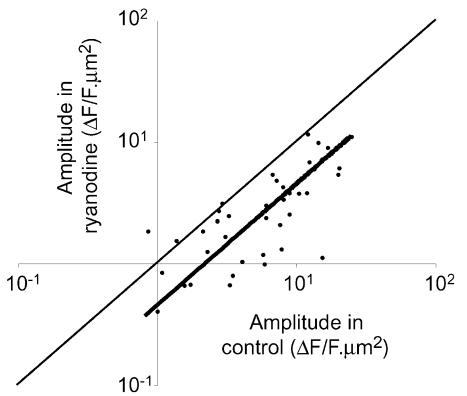

Figure 2. Intracellular Ca^{2+} stores and NCTs

The amplitudes of NCTs under control conditions and in the presence of ryanodine (10 μM) are shown. Each point represents the amplitude of NCTs occurring at a single neuroeffector junction. The thin line is a line of equivalence (where the amplitudes are unchanged), while the thick line is a linear curve fit (assuming that the curve passes through 0,0). *F* is the fluorescent intensity, in arbitrary units; Δ*F* is the change in *F* from its resting level.

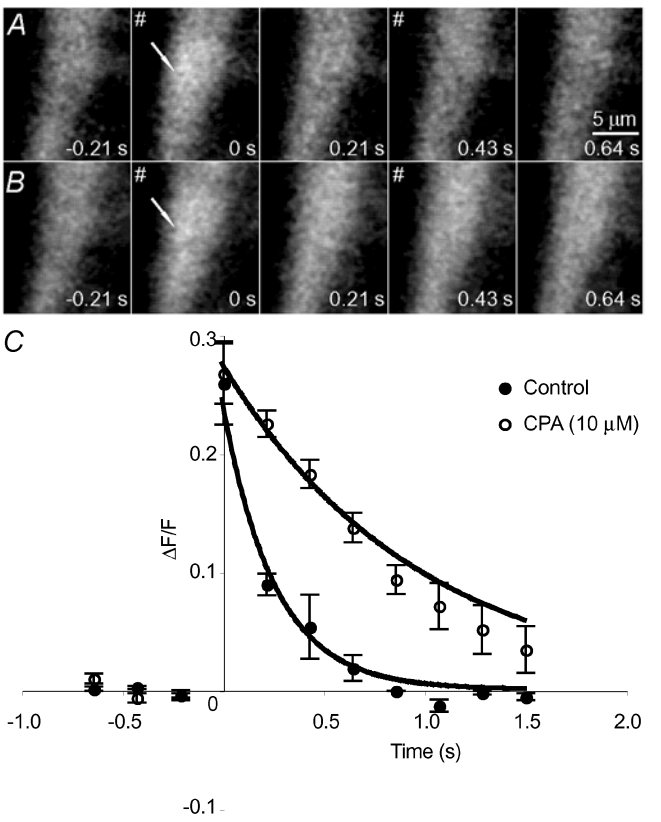

Figure 3. The rate of recovery of NCTs is slowed by CPA

A, a control recording from a smooth muscle cell during field stimulation (#). In this cell, NCTs arose at the location marked with an arrow with a probability of 0.06 per field stimulus. *B*, the same region in the presence of CPA (10 μM). Field stimuli still evoked NCTs with a similar probability (0.05 at this site), but the rate of the recovery of Ca^{2+} to its resting concentration is significantly slower. This is quantified for this junction in *C*. Points plotted are means ± S.E.M.

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Smooth muscle Ca^{2+} waves

Ca^{2+} waves arose spontaneously in a small number of smooth muscle cells (see Fig. 6 for an example). These arose from characteristic locations within the cell and propagated bidirectionally at an average speed of 5.8 ± 0.4 μm s^{-1} (n_{sm} = 3; n_p = 3). They travelled for 5–50 μm within the cells before collapsing. The frequency at which these waves occurred varied over time, and hence they could not be well characterised. In one cell only, waves arose from the same location as a cluster of NCTs, and were initiated upon nerve stimulation even in the presence of the α_1-adrenoceptor antagonist prazosin (100 nM). No intercellular Ca^{2+} waves were observed (n_p = 12).

DISCUSSION

Purinergic NCTs have now been identified in both the mouse vas deferens (Brain *et al.* 2002) and rat mesenteric arteries (Lamont & Wier, 2002; Lamont *et al.* 2003). NCTs are of physiological interest because of their potential role

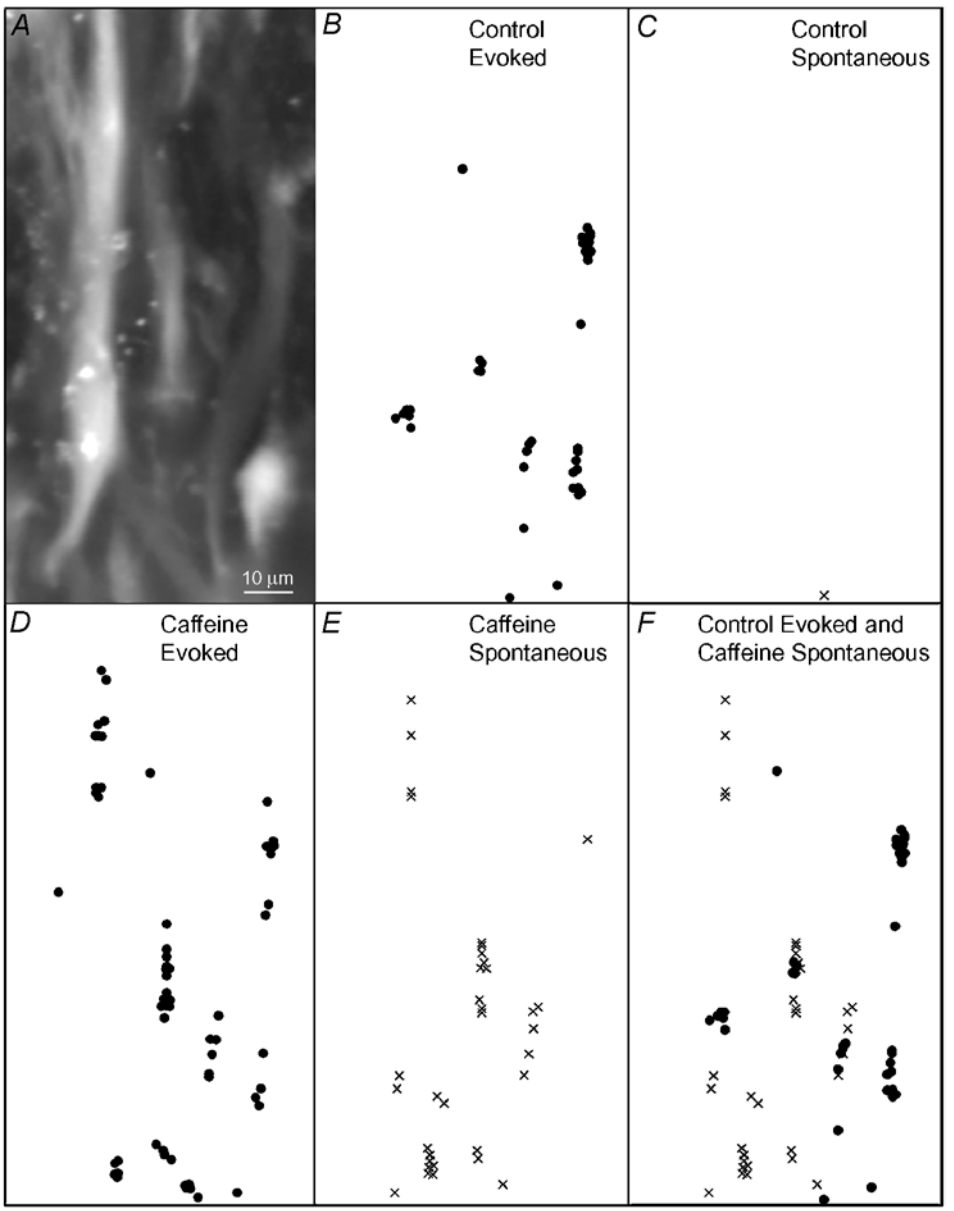

Figure 4. Caffeine evokes focal Ca^{2+} transients

A, images of several smooth muscle cells loaded with the Ca^{2+} indicator Oregon-BAPTA. *B–F* show maps (drawn to the same scale and position as the image in *A*) of the location of focal Ca^{2+} transients occurring immediately after field stimuli (dots; *B*, *D* and *F*), or without stimuli (crosses; frames *C*, *E* and *F*), at some time over 20 min of recording in control (*B*, *C* and *F*) or in the presence of caffeine (3 mM; *D*, *E* and *F*). Note that the evoked focal Ca^{2+} transients (NCTs) cluster in characteristic locations within parts of each cell (*B*). Caffeine induces spontaneous focal Ca^{2+} transients at additional locations (*E*; *F*, a composite of *B* and *E*).

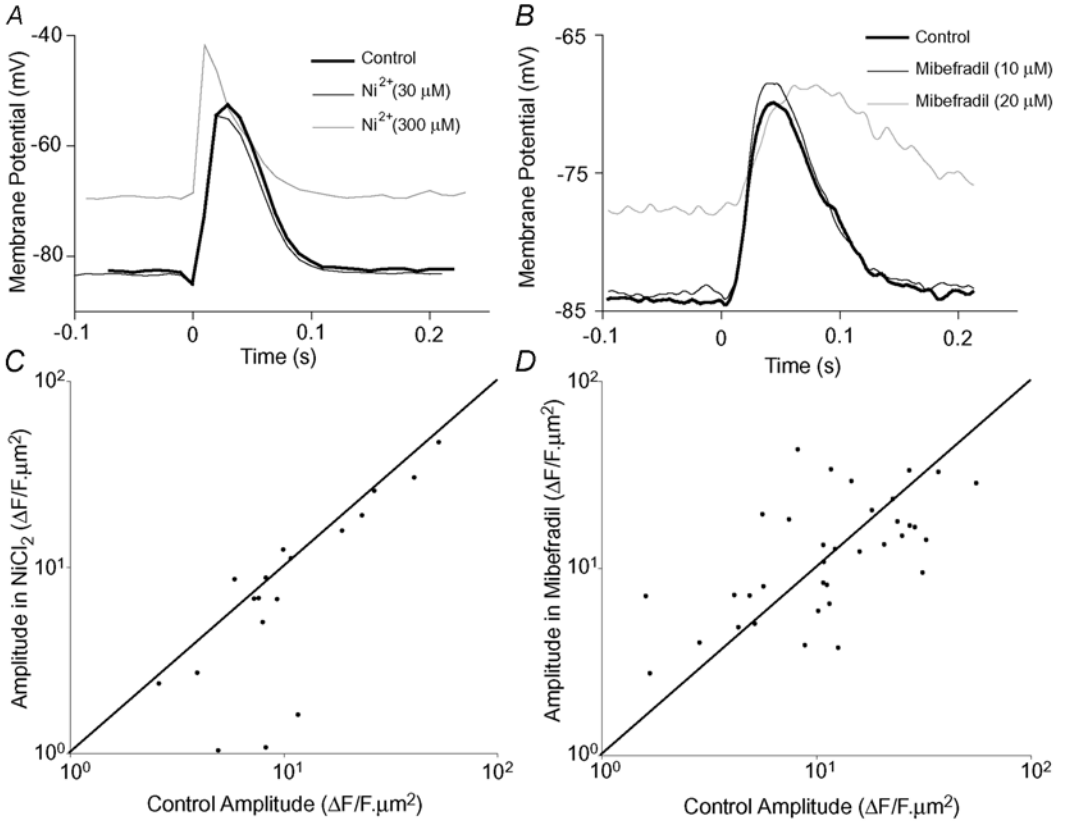

Figure 5. The effects of T-type Ca^{2+} channel blockers on EJPs and NCTs

A and *B* show the effect of Ni^{2+} or mibefradil (respectively) on EJPs during low frequency (0.33 Hz) stimulation. The recordings were zeroed with respect to the potential recorded by the microelectrode outside the cell at the end of each experiment. The sampling frequency in *A* is 100 Hz, while that in *B* is 4 kHz. The amplitudes of NCTs under control conditions and in the presence of either $NiCl_2$ (100 μM; *C*) or mibefradil (10 μM; *D*) are also shown. Each point represents the amplitude of NCTs occurring at a single neuroeffector junction. A line of equivalence (where the amplitudes are unchanged) is marked on each graph. At concentrations specific for T-type Ca^{2+} channel block, neither of these drugs significantly affects the amplitude of EJPs or NCTs.

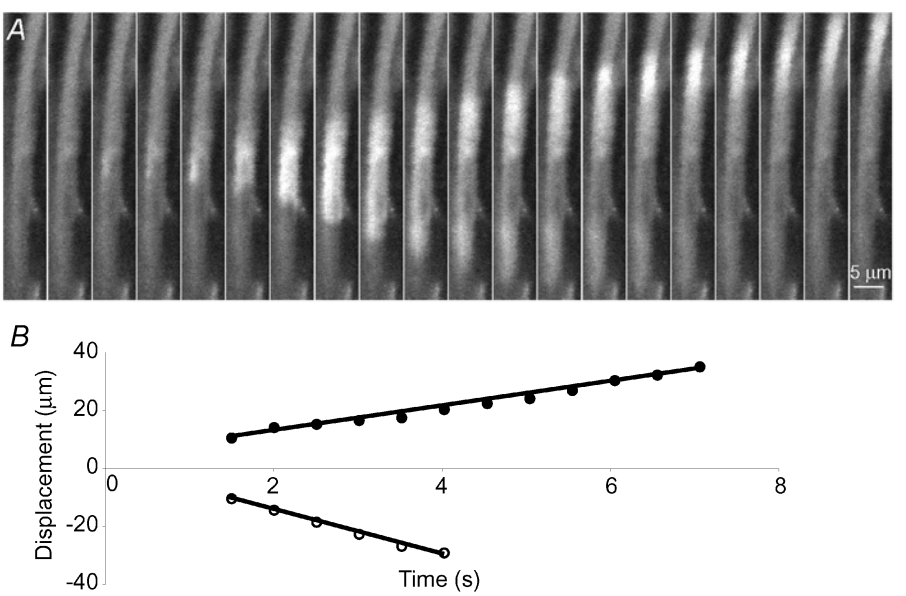

Figure 6. Spontaneous Ca^{2+} waves in a smooth muscle cell

A, an example of a cell in which Ca^{2+} waves arose spontaneously and propagated bidirectionally. The locations of the wave fronts are plotted in *B*. In this cell the average speeds of the waves were 4.4 μm s^{-1} (●) and 7.4 μm s^{-1} (○). *A* and *B* have the same time scale.

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in excitation–contraction coupling and as an optical tool for detecting the packeted release of neurotransmitters.

The initial observations of NCTs were made in smooth muscle cells filled with the dextran conjugate of the Ca^{2+} indicator Oregon Green 488 BAPTA-1. The present work confirms that the acetoxymethyl ester of the indicator can be loaded into these smooth muscle cells, and detects NCTs with a similar frequency to those reported with the dextran conjugate of the indicator (0.020 ± 0.002 compared to 0.019 ± 0.002 per junction; Brain *et al.* 2002).

In some smooth muscle cells from the mouse vas deferens, excitatory junction potentials (or currents) are not abolished by 10–20 μM α,β-methylene ATP (Allcorn *et al.* 1986; Liang *et al.* 2000), a $P2X_1$ receptor agonist which causes desensitisation and internalisation of $P2X_1$ receptors (Ennion & Evans, 2001). Given that EJPs do not occur in $P2X_1$ receptor knockout mice (Mulryan *et al.* 2000), it may be that α,β-methylene ATP achieves poor access to some junctions and hence does not abolish all NCTs.

It has previously been noted that NCTs precede local contraction of smooth muscle cells (Brain *et al.* 2002). That these local contractions remain in the presence of prazosin (100 nM) shows that the contractions do not require α_1-adrenoceptor activation and are generated by purinergic receptor activation. It is known that the L-type Ca^{2+} channel blocker nifedipine (1–10 μM) abolishes the purinergic component of contraction in the mouse vas deferens (Rae & Calixto, 1989; Cleary *et al.* 2003), suggesting that NCTs alone are not sufficient for coordinated contraction of the longitudinal layer of the mouse vas deferens. However, in rat mesenteric arteries NCTs contribute to the purinergic component of contraction (Lamont *et al.* 2003). Whether the local contraction subserves a physiological role in the mouse vas deferens, or whether the local $[Ca^{2+}]_i$ transient has a local regulatory function (such as coordinating the assembly of $P2X_1$ receptor clusters) are matters for further investigation.

The present results indicate that the local Ca^{2+} influx through P2X receptor clusters is amplified by CICR, as blocking CICR with ryanodine reduced the amplitude of NCTs. This assertion is plausible given that the Ca^{2+} permeability of $P2X_1$ receptors is large (Evans *et al.* 1996) and that the sarcoplasmic reticulum in many smooth muscle cells is closely associated with the plasma membrane (Devine *et al.* 1972; Gollasch *et al.* 1998; Lesh *et al.* 1998). CICR has previously been shown to augment the purinergic component of contraction in the rat vas deferens (Bourreau *et al.* 1991) and guinea-pig urinary bladder (without affecting the EJP; Hashitani *et al.* 2000), and to amplify whole-cell Ca^{2+} transients that follow the focal application of ATP to dissociated rat portal vein smooth muscle cells (Mironneau *et al.* 2001). It has recently been reported that NCTs in rat mesenteric artery smooth muscle cells are 'largely unaffected' by ryanodine, although a small but significant reduction (about 13 %) in the amplitude of NCTs was noted (Lamont & Wier, 2002). Hence, the extent to which intracellular Ca^{2+} stores contribute Ca^{2+} to NCTs may vary among different tissues.

Ryanodine can prevent CICR by preventing high conductance Ca^{2+} flux though ryanodine (RYR) receptors (Meissner, 1986), while CPA and thapsigargin are endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitors (for review, see Treiman *et al.* 1998) and may not prevent CICR if Ca^{2+} depletion in the ER is incomplete. Both CPA and thapsigargin slowed the rate at which Ca^{2+} returned to its resting concentration, implying that intracellular Ca^{2+} stores sequester at least some of the Ca^{2+} in NCTs. This effect was more pronounced with CPA. While thapsigargin is widely used as a Ca^{2+}-ATPase inhibitor, there are Ca^{2+}-ATPases that fill stores responsible for CICR that are resistant to thapsigargin (Bian *et al.* 1991; Tanaka & Tashjian, 1993). Furthermore, ER stores located close to the plasma membrane of glial cells and triggered by exogenously applied ATP are particularly sensitive to cyclopiazonic acid (Golovina & Blaustein, 2000). Such sub-plasmalemmal ('junctional') ER (Blaustein & Golovina, 2001) may be functionally adapted to link local neurotransmitter action with a local increase in Ca^{2+} concentration.

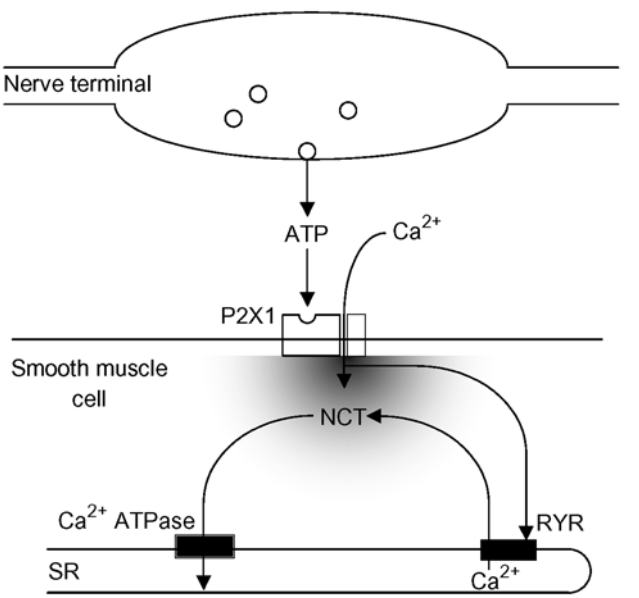

Figure 7. A model for the generation and sequestration of NCTs

Following a nerve terminal action potential there is only a small probability (about 0.02) that a packet of ATP will be released. This ATP acts on postjunctional $P2X_1$ receptors. The influx of Ca^{2+} through the P2X receptors is sufficient to cause a significant change in the local Ca^{2+} concentration, which is amplified by CICR from the RYR receptor. At least some of this Ca^{2+} is sequestered by the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase.

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The observation that focal $[Ca^{2+}]_i$ transients can be induced by caffeine demonstrates that Ca^{2+} 'sparks' can occur in mouse vas deferens, as in other (Bolton & Imaizumi, 1996; Kirber *et al.* 2001; Ohi *et al.* 2001) smooth muscle cells. Such Ca^{2+} sparks can be induced both at locations where NCTs could be evoked and at other sites, suggesting that not all intracellular Ca^{2+} stores are associated with functional P2X receptors. It is unlikely that caffeine increased the frequency of spontaneous transmitter release from otherwise silent junctions, as caffeine (10 mM) has no effect of the frequency of spontaneous EJPs in the rodent (guinea-pig) vas deferens (Ziogas *et al.* 1995).

In the present study mibefradil (10 μM) had no effect on the amplitude of excitatory junction potentials or on P_{NCT}, implying that T-type Ca^{2+} channels do not contribute to neurotransmitter release from nerve terminals or act postjunctionally to affect EJPs in the mouse vas deferens. This finding is consistent with the observations that neither mibefradil (up to 30 μM) nor Ni^{2+} (300 μM) had a significant effect on nerve stimulation-evoked contraction in the rat vas deferens (Xi & Angus, 2001; Xi *et al.* 2002). It is known that T-type Ca^{2+} channels contribute to EJPs in rat mesenteric arteries (Xi *et al.* 2002). It may be that this difference between tissues arises because of the relatively negative resting membrane potential in the mouse vas deferens (about −85 mV, compared to −60 mV in mesenteric arteries), so that a small depolarisation will not reach the threshold for activation of T-type Ca^{2+} channels. At a higher mibefradil concentration (20 μM) there was significant membrane potential depolarisation and a slowing of repolarisation. These actions of mibefradil are consistent with K^+ channel inhibition, which has been shown to occur in human myoblasts and other cells (Liu *et al.* 1999).

The observation that neither Ni^+ (100 μM) nor mibefradil (10 μM) affected the amplitude of NCTs implies that T-type Ca^{2+} channels do not contribute to the local Ca^{2+} influx that follows P2X receptor activation.

It is known that α_1-adrenoceptor agonists and noradrenergic transmission can induce or increase the frequency of Ca^{2+} waves in many smooth muscle cells (for examples, see Iino *et al.* 1994; Ruehlmann *et al.* 2000; Lee *et al.* 2001). The wave speed range varied from 20–90 μm s^{-1} in isolated smooth muscle cells of the rabbit inferior vena cava and was increased by exposure to an α-adrenergic receptor agonist (Ruehlmann *et al.* 2000). In the present study, spontaneous waves occurred even in the presence of prazosin, suggesting that they did not require the activation of α_1-adrenoceptors. The waves were also slower (at about 5 μm s^{-1}) than those previously reported.

The proposed model for the genesis and sequestration of NCTs is summarised in Fig. 7. It may be that the sequestration of Ca^{2+} by intracellular Ca^{2+} stores, following its entry through P2X receptors, is important in terminating the purinergic twitch. Ca^{2+} influx through P2X receptors may also charge intracellular Ca^{2+} stores for subsequent Ca^{2+} release, hence providing a mechanism for purinergic augmentation of noradrenergic neurotransmission.

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Supplementary material

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and contains material entitled:

Local twitches are associated with NCTs.