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Action of ryanodine on neurogenic responses in rat isolated mesenteric small arteries

[1]Robinder S. Garcha & Alun D. Hughes

Department of Clinical Pharmacology, Imperial College School of Medicine at St. Mary's, London W2 1NY

1 Rat mesenteric arteries (~250 μm) were set up in a single-channel isometric myograph designed to allow fluorescence measurements concurrent with field stimulation of intramural nerves. Vessels were loaded with 6 μM fura-2AM for 2 h and simultaneous recordings of neurogenic contraction (force) and intracellular calcium $[Ca^{2+}]_i$ were obtained. In other experiments, arteries were loaded with 1 μCi ml^{-1} [^{3}H]-noradrenaline (NA) for 30 min in order to measure release of [^{3}H]-NA in response to field stimulation to examine whether ryanodine directly inhibited neuronal release of NA.

2 Arteries were activated by single intermittent field stimulation or continuously to excite intrinsic sympathetic nerves, or by cumulative addition of noradrenaline (1 nM – 10 μM) to the bathing solution.

3 Pre-incubation with ryanodine markedly inhibited the contraction and $[Ca^{2+}]_i$ release in response to single-pulse nerve stimulation. Ryanodine also inhibited an early phasic component of the response to continuous field stimulation and reduced the rate of rise in force in response to continuous field stimulation. However, stable maximal contraction and $[Ca^{2+}]_i$ in response to continuous field stimulation as well as maximal responses to exogenous NA were unaffected. Release of [^{3}H]-NA in response to single intermittent field stimulation was not affected by ryanodine when compared to vehicle.

4 Our results suggest that brief intermittent activation of intramural sympathetic nerves increases $[Ca^{2+}]_i$ and contracts small arteries primarily by releasing Ca^{2+} from a ryanodine-sensitive intracellular store. In contrast, the stable rise in tone and $[Ca^{2+}]_i$ resulting from continuous nerve stimulation may largely depend on sources of Ca^{2+} other than the ryanodine-sensitive intracellular store.

Keywords: Nerve stimulation; ryanodine; intracellular calcium; vascular smooth muscle; arteries; mesenteric; electrical field stimulation

Introduction

A rise in $[Ca^{2+}]_i$ is generally considered to be the predominant signal leading to contraction of smooth muscle. The increase in $[Ca^{2+}]_i$ may arise from two sources; influx of extracellular Ca^{2+} and release of Ca^{2+} stored intracellularly (Himpens *et al*., 1995). The relative importance of these two sources can vary either depending on the stimulant or the blood vessel studied. Intracellular calcium stores are believed to be anatomically located in the endoplasmic (sarcoplasmic) reticulum. Ca^{2+} in these stores can be released by inositol 1,4,5-trisphosphate produced as a result of agonist-induced activation of phospholipase C (Berridge & Irvine, 1989), or pharmacologically by caffeine (Deth & Casteels, 1977). Ryanodine, a plant alkaloid, depletes Ca^{2+} from these stores (Smith *et al*., 1988) probably as a result of its ability to bind to and open Ca^{2+} permeable channels in the sarcoplasmic reticulum (Iino *et al*., 1988). Consequently, ryanodine has been widely used to study the functional importance of the intracellular store *in vitro*. In general, such studies indicate that release of Ca^{2+} from intracellular stores appears to make a large contribution to force generation in large 'conduit' arteries in response to exogenous agonists such as noradrenaline (NA) (Ashida *et al*., 1988). Previous studies with small resistance arteries (< 500 μm diameter) have shown that release of ryanodine-sensitive intracellular stores contributed little to force generation or the rise in $[Ca^{2+}]_i$ in response to exogenous noradrenaline in rat isolated small mesenteric arteries of a size considered important in the maintenance of peripheral vascular resistance (Garcha & Hughes, 1995). However, *in vivo* sympathetic nerves probably represent the major source of noradrenergic activation in the vasculature. Sympathetic nerve 'firing' in the vasculature is known to be complex; the concentration and temporal patterns of NA achieved in the vicinity of the vascular smooth muscle are likely to be highly variable so neural adrenergic activation of blood vessels may differ markedly from the application of exogenous NA (Bevan *et al*., 1989). In addition, in some vascular beds non-adrenergic neurotransmission may also make an important contribution to responses to sympathetic nerve activation.

We have therefore used rat isolated mesenteric small arteries to investigate what part ryanodine-sensitive intracellular stores contribute to the neurogenic-induced rise in $[Ca^{2+}]_i$ and tone in response to sympathetic nerve stimulation.

Methods

Experimental protocol

Male Sprague-Dawley rats (200 – 300 g) were used in all studies. The animals were killed by a blow to the neck and the mesentery isolated. Mesenteric resistance arteries were dissected (~250 μm internal diameter) and mounted as ring segments in an isometric myograph (Mulvany & Halpern, 1977). All experiments were conducted in a modified Krebs physiological saline solution (PSS) (composition shown below). Arteries were set up in a single-channel myograph dedicated to simultaneous fluorescence and tension measurements. The vessels were allowed to equilibrate for 1 h and then set at a 'normalized' internal circumference 0.9 L_{100} estimated to be 0.9 times the circumference they would maintain if relaxed and exposed to 100 mmHg transmural pressure. This was calculated for each individual vessel on the basis of the passive length-tension characteristics of the artery and the Laplace relationship (Mulvany & Halpern, 1977). This procedure optimized active force generation by these vessels and the internal diameters referred to were derived from this calculation. Before the studies were begun, vessel viability was assessed by exposing arteries to noradrenaline (10 μM) or PSS containing

[1] Author for correspondence.

118 mM KCl (equimolar substitution for NaCl; KPSS). Vessels which failed to produce tension reproducibly equivalent to more than 100 mmHg effective pressure (by Laplace) in response to these stimulants were discarded. Vessels used for field stimulation studies were not exposed to KPSS as this depressed subsequent responses to nerve stimulation (data not shown).

Nerve-stimulation

Arteries were mounted on wires sited between two 200 μm thick platinum wire electrodes and square wave field pulses (15 V) were passed between the electrodes in a monophasic manner, either continuously or as a single pulse intermittently. The stimulation parameters were controlled with a Grass S48 stimulator. Responses elicited in this way could be inhibited by 0.1 μM tetrodotoxin, and were therefore considered to be neurogenic. Contraction in response to a single field stimulation (15 V, 0.3 ms duration) was almost completely abolished by pre-incubation for 10 min with 10 μM phentolamine (control response $=6.2\pm1.3$ Nm^{-1}, response in presence of phentolamine $=0.2\pm0.1$ Nm^{-1}; $n=4$) implying that it was predominantly due to activation of α-adrenoceptors.

Separate studies were conducted to examine the effect of Ca^{2+} store depletion on transient 'twitch' responses to intermittent single pulse or continuous field stimulation. Single pulse or continuous stimulation experiments were performed in separate vessels. Twitch responses were evoked by a single pulse of electrical field stimulation (15 V) of 0.3 ms duration applied at 50 s intervals; a total of 8 pulses were applied. After wash-out and recovery the vessel was then incubated with ryanodine (10 μM, 1 h) or vehicle for 1 h and the stimulation protocol repeated following washout of ryanodine, the effects of which are irreversible in these vessels (Garcha & Hughes, 1995). Responses to the first 3–4 pulses tended to show a progressive increase in response but the responses to the last 4 pulses were stable and these were therefore averaged for the purposes of calculations. Continuous field stimulation was applied as a cumulative frequency-response curve (15 V, 1–16 Hz, 0.3 ms pulse width) with stimulation being maintained for 50 s at each frequency. The response to continuous field stimulation was taken to be the maximum stable response to stimulation. The initial transient response corresponding to the commencement of stimulation was excluded from this analysis. Similar studies were performed after the vessel was incubated with ryanodine for 1 h and the curve repeated. Since the effect of ryanodine in these vessels is irreversible the sequence of application could not be altered randomly to exclude time and/or order effects, so vehicle-only time-controls were also performed in the absence of ryanodine.

The effect of ryanodine on exogenous noradrenaline (1 nM–10 μM) was also investigated in some of the same vessels. Noradrenaline concentration-response curves (1 nM–10 μM) were performed in PSS, in the presence of cocaine 1 μM, propranolol 1 μM and N$^{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME) 10 μM to inhibit catecholamine uptake, β-adrenoceptors and endothelium-derived relaxing factor (EDRF), respectively. The concentration-response curve to noradrenaline was performed following preincubation with ryanodine (10 μM, 1 h) or vehicle as previous studies have demonstrated that concentration-dependent responses to noradrenaline do not show tachyphylaxis under these conditions (Garcha & Hughes, 1995).

Fluorescence measurements of intracellular $[Ca^{2+}]_i$

Simultaneous mesurement of tension and $[Ca^{2+}]_i$ was performed with the fluorescent calcium indicator fura-2 (Jensen *et al*., 1992). Arteries were incubated with 6 μM fura-2/AM for 2 h in PSS at 37°C. After being loaded, vessels were thoroughly washed to remove free fura-2AM and allowed to equilibrate in PSS for 30 min before fluorescence measurements were made. Fluorescence was measured by use of a Deltascan spectrofluorimeter (Photon Technology International Inc., South Brunswick, NJ, U.S.A.) connected to an Axiovert 35 fluorescence microscope (Carl Zeiss Oberkochen, Germany) with only quartz objectives (Ultrafluor $\times$ 10). $[Ca^{2+}]_i$ was assessed on the basis of the ratio of fluorescence emission measured at 510 ± 5 nm which was evoked by excitation at 340 nm and 380 nm. Emission signals and force were measured simultaneously at 4 Hz and acquired on-line by use of an A/D interface (Photon Technology International Inc., South Brunswick, NJ, U.S.A.) connected to an IBM AT PC. Data were stored on an optical disc and later analysed off-line by use of commercially available software (Photon Technology International Inc., South Brunswick, NJ, U.S.A.).

Experiments were not calibrated to absolute values for $[Ca^{2+}]_i$ (Grynkiewicz *et al*., 1985), since previous experiments (Garcha & Hughes, 1994) have shown calibration with ionomycin to be inconsistent in these vessels and could be associated with leakage of dye leading to spurious values for R_{min} and R_{max}. Consequently changes in $[Ca^{2+}]_i$ in response to sympathetic nerve stimulation were normalized by expressing them as percentage change in peak ratio of 340/380 nm signal induced by exogenous NA (10 μM). For noradrenaline concentration-response experiments changes in $[Ca^{2+}]_i$ were normalized by expressing them as percentage change in peak ratio of 340/380 nm signal induced by depolarization with KPSS as previously described (Garcha & Hughes, 1994).

[^{3}H]-NA measurements

Release of [^{3}H]-NA was measured to examine whether ryanodine might affect neuronal release of NA. For these studies, arteries (length $=2$ mm) mounted in a myograph as for tension/$[Ca^{2+}]_i$ studies were loaded with 1 μC ml^{-1} [^{3}H]-NA

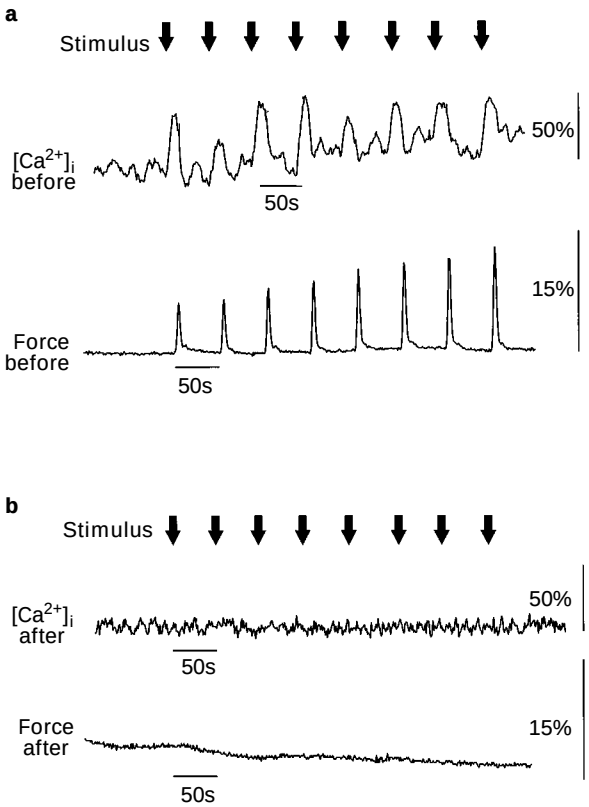

Figure 1 Example traces showing control before (a) and the effect of ryanodine (10 μM, 1 h) pre-exposure (b) on intracellular calcium ($[Ca^{2+}]_i$; %) and force production in response to single-pulse nerve stimulation. Eight single pulses (15 V, 0.3 ms) were applied with 50 s intervals between pulses as indicated above the traces. Calibration bars indicate % of response to 10 μM noradrenaline. Traces are representative of 4 similar experiments.

(100 nmol) for 30 min in PSS (Cena *et al*., 1985). Following incubation, vessels were washed three times and incubated with ryanodine (10 μM, 1 h) in PSS ($n=6$), or in PSS alone for 1 h (control, $n=8$). The bath content (5 ml) was collected at 5 min intervals over 80 min. Samples collected over the time period 0–45 min after wash-out were discarded and the 3 samples collected at 50, 55 and 60 min were used as mean [^{3}H]-NA release before stimulation. The stimulation period was the 5 min period, 60–65 min after wash-out of ryanodine or vehicle, single pulses of 15 V, 0.3 ms duration were applied with a 50 s interval between each pulse; as used for contraction/$[Ca^{2+}]_i$ studies, the effects of ryanodine are irreversible in these vessels (Garcha & Hughes, 1995). Following termination of nerve stimulation, the samples collected during time-period 70, 75 and 80 min served as mean [^{3}H]-NA release after stimulation.

Scintillant was added to the 5 ml samples and they were counted in a Packard 1700 liquid scintillation analyser. At the end of the experiment, arteries were unmounted and dissolved in 0.5 ml of Tissuesol, scintillant added and the sample counted. [^{3}H]-NA release in 5 min was expressed as fractional release (i.e. % of total tissue radioactivity). Total [^{3}H]-NA in the vessel sample at the end of the experiment was 10 ± 2 nmol (51000 ± 9000 c.p.m.); $n=14$.

Statistics

Values are expressed as mean $\pm$ s.e.mean. Statistical differences between two means were determined by Student's t test for paired data or unpaired data as appropriate. Concentration-response data were fitted to a logistic function by non-linear regression (Garcha & Hughes, 1994) and EC_{50}, the concentration of drug producing half-maximal response, calculated. Concentration-response data were compared in terms of EC_{50} and maximum response by use of a t test for paired data. Non-linear regression was carried out on an IBM PC compatible computer using commercially available software (Inplot 4.0, GraphPAD Software, CA, U.S.A.). Peak force and peak $[Ca^{2+}]_i$ were defined as the maximum values attained following drug application; the time of peak force did not necessarily correspond to the time of peak $[Ca^{2+}]_i$. The effect of drug treatment on peak force was compared by a Wilcoxon test for paired data. A value of $P<0.05$ was considered significant.

Drugs and solutions

The composition of the physiological saline solution (PSS) was (mM): NaCl 118, KCl 4.7, $CaCl_2.6H_20$ 2.5, $MgSO_4.7H_20$ 1.17, $NaHCO_3$ 25.0, $NaH_2PO_4.2H_20$ 1.0, Na_2EDTA 0.03 and glucose 5.5) maintained at 37°C and aerated with 95% oxygen/5% carbon dioxide. Noradrenaline, phentolamine, propranolol, and N$^{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma (Poole, Dorset, U.K.). Ryanodine was purchased from Calbiochem (CA, U.S.A.) and fura-2AM was purchased from Molecular Probes (Oregon, U.S.A.). Cocaine was purchased from Hillcross Pharmaceuticals (Burnley, U.K.). Tissuesol NCS was purchased from Amersham International (Amersham, U.K.). [7-^{3}H]-(−)-noradrenaline, was obtained from DuPont Merck (Wilmington,

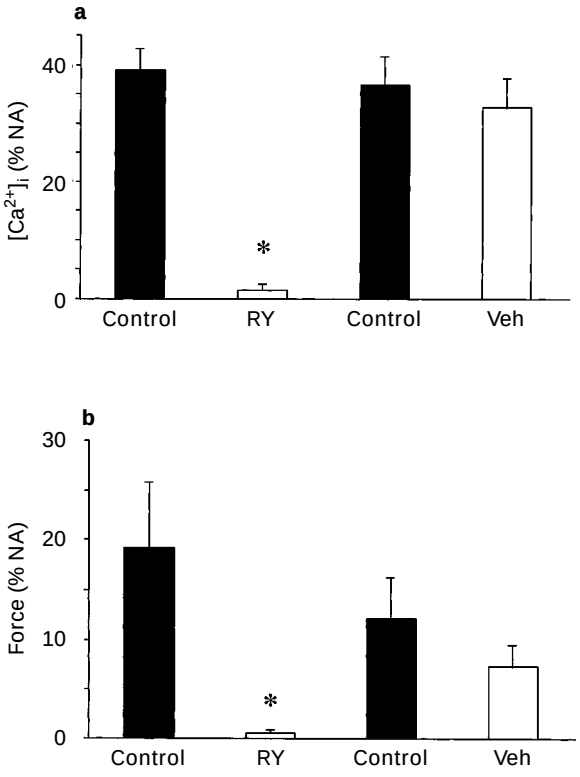

Figure 2 The effect of ryanodine on $[Ca^{2+}]_i$ and force in rat isolated mesenteric small arteries. (a) Peak rise in intracellular calcium ($[Ca^{2+}]_i$) in response to single pulse field stimulation before (control) and after ryanodine pre-incubation (RY; 10 μM, 1 h) or vehicle (Veh). (b) Peak force in response to single pulse field stimulation after ryanodine (RY; 10 μM, 1 h) or vehicle compared with respective controls before addition of drug. Columns represent mean $\pm$ s.e.mean of % control response to 10 μM noradrenaline (% NA), $n=4$ in all cases. *Indicates $P<0.05$ compared with the time-matched vehicle value.

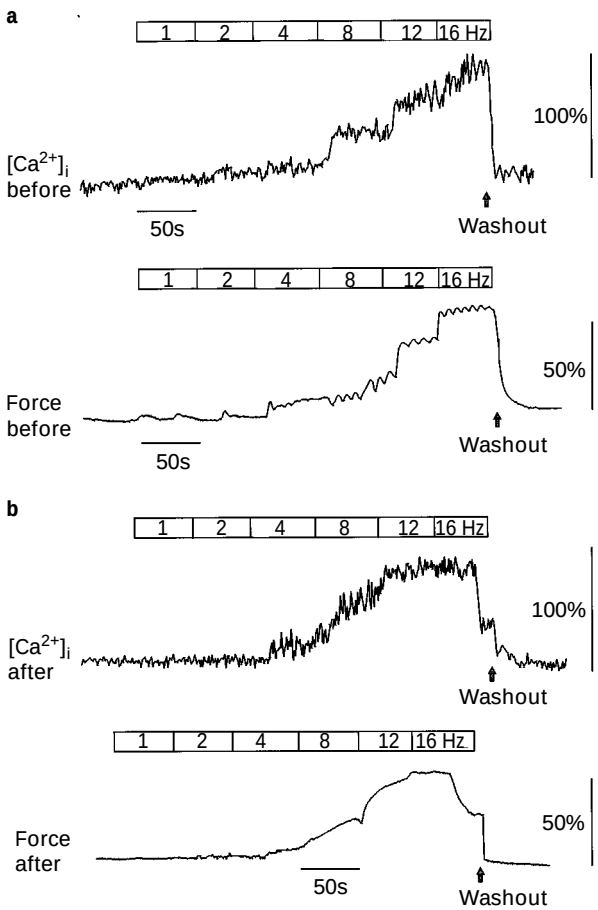

Figure 3 Example traces showing the effect of ryanodine (10 μM, 1 h) pre-exposure on intracellular calcium ($[Ca^{2+}]_i$) and force production in response to continuous nerve stimulation. Stimulation was applied as a cumulative frequency-response curve (15 V, 1–16 Hz, 0.3 ms pulse width) as indicated by the markers with stimulation applied for 50 s at each frequency. Calibration bars indicate % of response to 10 μM noradrenaline. Traces are representative of 4 similar experiments. (a) Measurement before ryanodine, (b) those after treatment with ryanodine.

DE, U.S.A.). Fura-2AM was made up in 25 μl dimethylsulphoxide (DMSO) Cremophor-EL/Pluronic F-127. The final concentration of DMSO (0.5%), Cremophor-EL (0.1%) and Pluronic F-127 (0.002%) in PSS did not affect basal tone or the contractile response of vessels.

Results

Both single pulse and continuous nerve stimulation induced contraction and an associated increase in $[Ca^{2+}]_i$ in rat isolated mesenteric arteries. A single pulse of field stimulation produced a rapid, monophasic contraction and rise in $[Ca^{2+}]_i$, both typically lasting 2–4 s (Figure 1). The average response to intermittent single pulse stimulation corresponded to $13 \pm 2\%$ of maximal force in response to 10 μM noradrenaline ($n=4$) and a rise in $[Ca^{2+}]_i$ which was $34 \pm 3\%$ of the maximal rise in $[Ca^{2+}]_i$ in response to 10 μM noradrenaline ($n=4$). Ryanodine treatment markedly attenuated both force and the rise in $[Ca^{2+}]_i$ in response to intermittent single pulse nerve stimulation compared with the time-matched control (Figures 1 and 2) ($P<0.05$). In control studies, after 1 h incubation with vehicle responses were slightly reduced but this was not statistically significant.

The contractile response to continuous nerve stimulation was usually biphasic: an initial prompt transient contraction followed by a sustained phase reaching a maximum in less than 1 min, a similar biphasic response of $[Ca^{2+}]_i$ to continuous nerve stimulation was less often seen (Figure 3). The maximal response to field stimulation, attained with 16 Hz, was $58 \pm 2\%$ of the maximal contraction ($n=4$) and $112 \pm 13\%$ of the maximal rise in $[Ca^{2+}]_i$ ($n=4$) to exogenous NA. Ryanodine abolished the initial phasic contraction in 3 out of 4 vessels in response to continuous nerve stimulation (Figure 3), and the rate of rise in force was slower following ryanodine (Table 1). Ryanodine had no effect on the time taken to achieve 50% of peak $[Ca^{2+}]_i$ in response to continuous stimulation (Table 1). Ryanodine treatment did not significantly inhibit maximal sustained force and $[Ca^{2+}]_i$ in response to continuous field stimulation. Following ryanodine treatment, maximal sustained response to continuous stimulation, attained with 16 Hz, was $51 \pm 2\%$ of the maximal contraction to exogenous NA ($n=4$) and $91 \pm 10\%$ of the maximal rise in $[Ca^{2+}]_i$ to

Table 1 The effect of ryanodine on the time taken to achieve 50% of peak force and peak $[Ca^{2+}]_i$ following activation by continuous field stimulation at 8 Hz

	Pre-treatment		*Post-treatment*	
	$t_{\frac{1}{2}}$ *force* (s)	$t_{\frac{1}{2}}[Ca^{2+}]_i$ (s)	$t_{\frac{1}{2}}$ *force* (s)	$t_{\frac{1}{2}}[Ca^{2+}]_i$ (s)
Ryanodine	3 ± 1	4 ± 1	24 ± 2*	6 ± 1
Control	4 ± 1	4 ± 1	4 ± 1	5 ± 1

Data are means $\pm$ s.e.mean of 3–4 observations. *$P<0.05$.

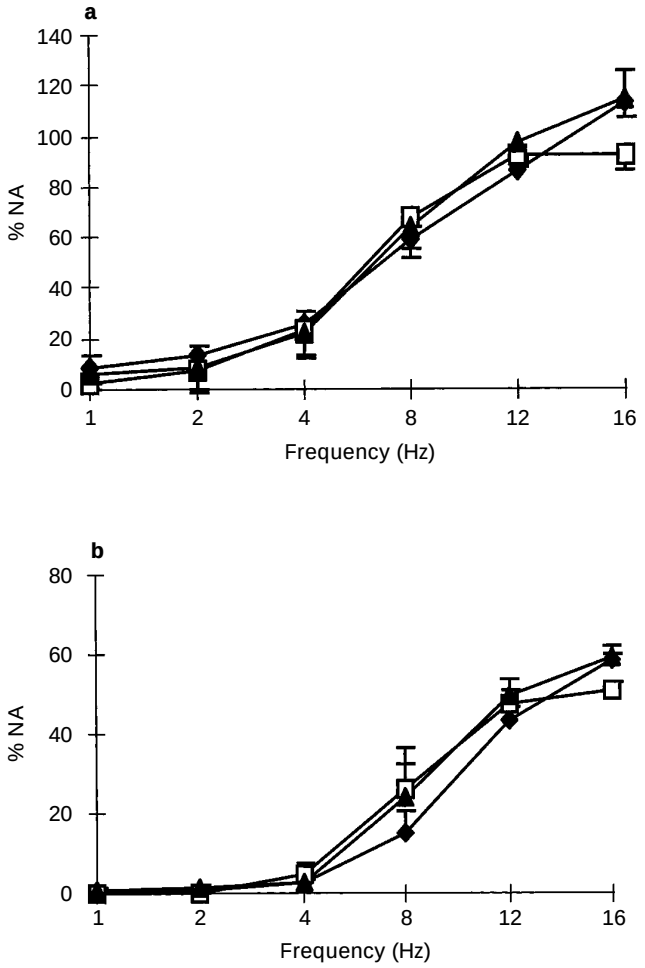

Figure 4 Frequency-response curve for the rat mesenteric artery (1–16 Hz, continuous stimulation). (a) Maximum sustained rise in intracellular calcium ($[Ca^{2+}]_i$) before (◆) (pooled data) and following ryanodine pre-exposure (□) (10 μM, 1 h) and vehicle (▲). (b) Maximum sustained force before (◆) (pooled data) and following ryanodine pre-exposure (□) (10 μM, 1 h) and vehicle (▲). Responses are % response to 10 μM noradrenaline (% NA). Each point is the mean of 4 vessels; vertical lines show s.e.mean.

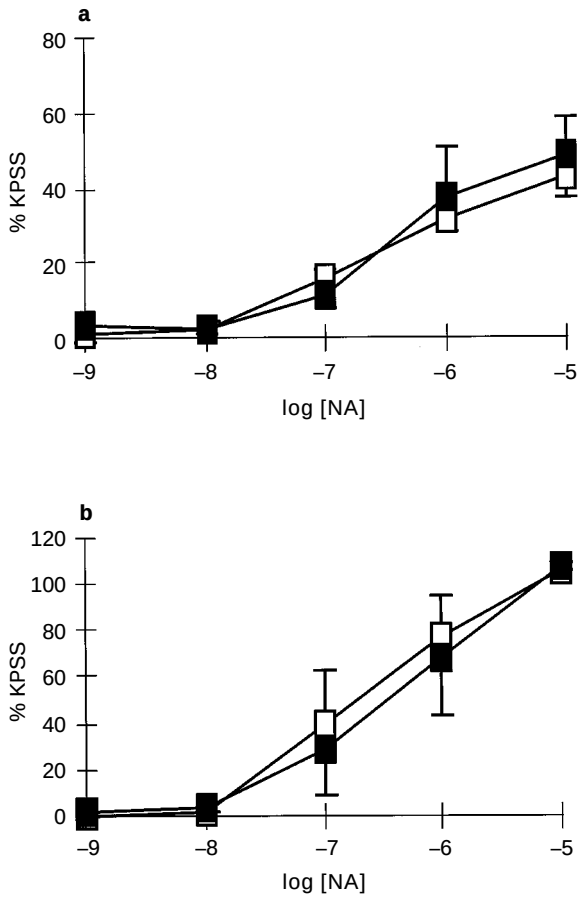

Figure 5 Concentration-response curves to noradrenaline (NA; 1 nM–10 μM) for the rat mesenteric artery. (a) Maximum rise in intracellular calcium ($[Ca^{2+}]_i$) following ryanodine pre-exposure (□) (10 μM, 1 h) and control (■). (b) Maximum force following ryanodine pre-exposure (□) (10 μM, 1 h) and control (■). Responses are % response to depolarizing potassium solution (% KPSS). Each point is the mean of 4 vessels; vertical lines show s.e.mean.

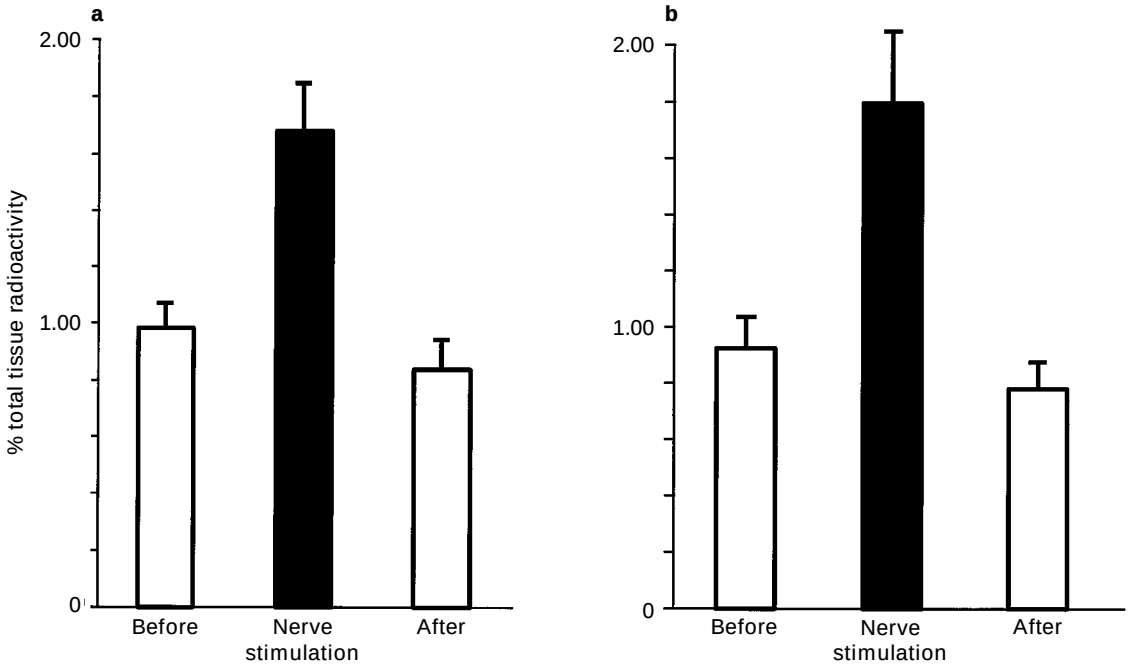

Figure 6 Release of [^{3}H]-NA in response to nerve stimulation before and after ryanodine pre-exposure (10 μM, 1 h) ($n=6$) (a) compared with vehicle ($n=8$) (b). Data were calculated as [^{3}H]-NA release during 5 min divided by total tissue [^{3}H]-NA $\times$ 100%. Columns represent mean $\pm$ s.e.mean. Before = mean [^{3}H]-NA release at time 50, 55 and 60 min after initial wash-out. Nerve stimulation = mean [^{3}H]-NA release on nerve stimulation over the time period 60–65 min after initial wash-out during which field stimulation with single pulses (15 V, 0.3 ms) were applied every 50 s. After = mean [^{3}H]-NA release following termination of nerve stimulation at time 70, 75 and 80 min after initial wash-out.

exogenous NA (both NS, $n=4$) (Figure 4). As has been previously shown (Garcha & Hughes, 1995), the concentration-response relationship to exogenous NA (1 nM – 10 μM) was unaffected by ryanodine. Control values for maximal contraction and rise in $[Ca^{2+}]_i$ were $108 \pm 3\%$ and $43 \pm 6\%$, respectively, in the absence of ryanodine, and $106 \pm 4\%$ and $48 \pm 10\%$, respectively, following ryanodine ($n=4$; values expressed as % KPSS response) (Figure 5).

The release of [^{3}H]-NA in response to nerve stimulation was not affected by ryanodine ($n=6$) when compared to vehicle ($n=8$) (Figure 6).

Discussion

These studies confirm that electrical field stimulation causes contraction and a rise in $[Ca^{2+}]_i$ in rat isolated mesenteric resistance arteries as was previously described by Jensen (1995). The sensitivity to tetrodotoxin and the near total inhibition of responses to field stimulation by phentolamine imply that the responses to electrical field stimulation were largely due to activation of perivascular sympathetic nerves leading to release of NA and activation of postjunctional α-adrenoceptors. Evidence in the literature regarding the contribution of α-adrenoceptors to nerve-evoked contraction in rat mesenteric arteries is conflicting. Sjöblom-Widfeldt *et al.* (1990) showed that $\sim$80% of the response to a single twitch remained after prazosin or phenoxybenzamine. In contrast, Furness & Marshall (1974) showed that phentolamine completely blocked responses of rat mesenteric arteries to continuous field stimulation. Nilsson (1984) showed that in rat mesenteric small artery responses to continuous field stimulation were blocked by phentolamine and prazosin, although occasionally up to 15% of the response remained after phentolamine. Similarly, Jensen (1995) found that phentolamine almost completely abolished both the rise in $[Ca^{2+}]_i$ and force in response to continuous field stimulation in rat mesenteric small arteries. Why such discrepancies exist is unclear since in most of these studies the same strain of rat (Wistar) of similar weight ($\sim$250 g) has been used, possibly small differences in arterial size or precise site of origin of vessel underlie these conflicting observations.

Previous detailed analysis of the properties of the innervation in the vasculature of the rat have shown that the small resistance arteries have the greatest innervation density and the maximal neurogenic response in these arteries is comparable in magnitude to the maximal response to exogenous NA (Nilsson *et al.*, 1986). Although differences between neurogenic responses to single-pulse and continuous nerve stimulation have been described in various blood vessels, these have mainly shown differences in the magnitude of contraction or the release of co-transmitters under different parameters of stimulation (Nilsson *et al.*, 1985 (single/continuous); Kennedy *et al.*, 1986 (short pulse burst/continuous); Sjöblom-Widfelt *et al.*, 1990 (single/continuous)). Release of intracellular Ca^{2+} has previously been proposed to be important in neural regulation of rat mesenteric artery on the basis of the inhibitory effects of caffeine (Nielsen & Mulvany, 1990); while in rat isolated tail artery smooth muscle cells, ryanodine treatment prevented $[Ca^{2+}]_i$ oscillations and subsequent propogation of a Ca^{2+} wave in response to nerve stimulation (Iino *et al.*, 1994).

In the present study, comparison of neurogenic responses of rat mesenteric arteries when activated by single-pulse and continuous stimulation indicate that the nature of the stimulation influences the source of Ca^{2+} utilized. Our data cannot exclude the possibility that Ca^{2+} store release is coupled to Ca^{2+} influx but do suggest that release of intracellular stores plays a primary role in the response to brief neural activation, as ryanodine virtually abolished both the contraction and the associated increase in $[Ca^{2+}]_i$ in response to single-pulse nerve stimulation. Ryanodine also significantly slowed the rate of rise in force in response to continuous field stimulation. The rate of rise in $[Ca^{2+}]_i$ was also slowed by ryanodine, though this difference did not achieve statistical significance, possibly reflecting poorer temporal resolution of the more rapid Ca^{2+} signals in arterial smooth muscle. It is possible that ryanodine also influenced spatial or temporal patterns of the Ca^{2+} signal in smooth muscle cells as previously described by Iino *et al.* (1994), though our study could not address this question.

Nevertheless, in mesenteric resistance arteries, since the ultimate level of $[Ca^{2+}]_i$ and tone achieved as a result of continuous nerve stimulation and exogenous noradrenaline were unaffected by ryanodine treatment, it seems likely that under these conditions the steady state contractile response is largely dependent on sources other than the intracellular store, such as influx of Ca^{2+}. Neurogenic responses of isolated arteries are inhibited by removal of extracellular Ca^{2+} (Sjöblom-Widfeldt & Nilsson, 1989). However, it is difficult to distinguish the effects of Ca^{2+} removal on neural release of transmitter (Kirpekar *et al*., 1975) from actions on the contractile process under these conditions. In rabbit isolated ear artery calcium channel antagonists have been shown to inhibit neurogenic responses without inhibiting release of [^{3}H]-noradrenaline from sympathetic nerves (Kajiwara & Casteels, 1983). Such a key role for voltage-operated calcium channels would be in keeping with its major role in mediating the increase in tone and $[Ca^{2+}]_i$ in rat mesenteric arteries following addition of exogenous noradrenaline (Nilsson *et al*., 1994). However, other studies have failed to be able to exclude prejunctional actions of calcium antagonists in causing a reduction in the response to nerve stimulation (Suzuki *et al*., 1982; Kuriyama *et al*., 1983). Our experiments indicate that ryanodine does not influence neural release of [^{3}H]-NA. This finding is consistent with previous studies showing little or no effect of ryanodine on noradrenaline release from the vas deferens (Bourreau, 1996) and cardiac sympathetic nerves (Gurnett *et al*., 1993). Therefore it seems likely that the inhibitory effect of ryanodine on single pulse stimulation represents an effect on smooth muscle rather than some action on transmitter release.

Our data suggest that the physiological importance of the intracellular Ca^{2+} store to neurogenic tone may depend on the characteristics of neural activation *in vivo*. Early studies suggested that autonomic neurones fired at a low average frequency which rarely exceed 10 Hz (Folkow, 1952). More recently, electrophysiological recordings in man have revealed that this is a net value and impulse frequency is not continuous, but occurs in bursts of high frequencies separated by quieter periods (Wallin, 1987). Stimulation with an irregular impulse pattern has been shown to induce greater responses (Nilsson *et al*., 1985; Hardebo, 1992) that are better sustained than during continuous stimulation (Andersson, 1983). It is possible that different patterns of Ca^{2+} utilization could contribute to some of these effects.

In summary, our data indicate that in contrast to the response to continuous field stimulation or α-adrenoceptor activation by exogenous agonist, the release of Ca^{2+} from a ryanodine-sensitive store appears to play a major role in the response to brief sympathetic nerve activation in rat mesenteric arteries.

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