Ca^{2+}-induced Ca^{2+} release mediates Ca^{2+} transients evoked by single action potentials in rabbit vagal afferent neurones

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1. Standard intracellular recording techniques with 'sharp' micropipettes were used to evoke action potentials (APs) in acutely dissociated adult nodose neurones.

2. APs induced a transient increase in $[Ca^{2+}]_i$ (a calcium transient), recorded with fura-2, that was dependent upon $[Ca^{2+}]_o$ and the number of APs. Over the range of one to sixty-five APs, the relation between the amplitude of the calcium transient and the number of APs was well fitted by a rectangular hyperbola ($\chi^2 = 3{\cdot}53$, $r = 0{\cdot}968$). From one to four APs, the calcium transient–AP relation can be described by a line with a slope of 9·6 nM AP^{-1} ($r = 0{\cdot}999$).

3. Charge movement corresponding to Ca^{2+} influx evoked by a single AP was $39 \pm 2{\cdot}8$ pC (mean ± S.E.M.) and did not change significantly during trains of one to thirty-one APs ($P < 0{\cdot}05$).

4. Caffeine (10 mM), a known agonist of the ryanodine receptor, produced an increase in $[Ca^{2+}]_i$. The caffeine-induced rise in $[Ca^{2+}]_i$ was attenuated (by > 90%) by lowering $[Ca^{2+}]_o$, and by ryanodine (10 μM), 2,5-di(*t*-butyl)hydroquinone (DBHQ, 10 μM), or thapsigargin (100 nM).

5. Neurones incubated with ryanodine, DBHQ or thapsigargin required at least eight APs to evoke a detectable calcium transient. These reagents did not significantly affect Ca^{2+} influx ($P < 0{\cdot}05$). In the presence of these inhibitors, the calcium transient–AP relation exhibited slopes of 1·2, 1·1 and 1·9 nM AP^{-1} for ryanodine, DBHQ and thapsigargin, respectively. When compared with the slope of 9·6 nM AP^{-1} in non-treated neurones, it appears that Ca^{2+} influx produced by a single AP is amplified by *ca* 5- to 10-fold.

Calcium can regulate neuronal excitability through direct and indirect mechanisms. Activation of voltage-dependent Ca^{2+} channels (VDCCs) in plasma membranes can lead directly to changes in membrane excitability (Miller, 1987; Cheek, 1989; Marty, 1989). On the other hand, release of Ca^{2+} from intracellular stores initiated by activation of VDCCs or by agonists acting on plasma membrane receptors represents a mechanism whereby Ca^{2+} can produce alterations in neuronal excitability indirectly (Thayer, Perney & Miller, 1988). At least two intracellular Ca^{2+} stores have been distinguished in neurones, an inositol 1,4,5-trisphosphate-releasable store (Berridge & Irvine, 1989) and a caffeine- and ryanodine-sensitive store that is involved in Ca^{2+}-induced Ca^{2+} release (CICR) (reviewed by Kuba, 1994).

CICR is a well-characterized process in skeletal and cardiac muscle, where it functions to prolong and amplify the rises in $[Ca^{2+}]_i$ elicited by physiological stimuli (Fabiato, 1983; Endo, 1985). Several criteria have been developed to establish the existence of CICR in cells. These include: (i) a rise and decay of $[Ca^{2+}]_i$, the Ca^{2+} transient, following an action potential (AP) or a step voltage command, that persists orders of magnitude longer than the time course of the Ca^{2+} current initiated by these stimuli; (ii) a calcium transient that is greater than can be accounted for by electrically stimulated Ca^{2+} influx across the plasma membrane; and (iii) a caffeine-sensitive intracellular Ca^{2+} store that is dependent upon the concentration of extracellular Ca^{2+} and modified by Ca^{2+}-ATPase inhibitors (e.g. thapsigargin), or by CICR channel modulators (e.g. ryanodine). Through the use of some or all of these criteria, CICR has been characterized in a variety of peripheral neurones (Kuba, Morita & Nohmi, 1983; Lipscombe, Madison, Poenie, Reuter,

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Tsien & Tsien, 1988; Hua, Nohmi & Kuba, 1993; Yoshizaki *et al.* 1995; Shmigol, Verkhratsky & Isenberg, 1995) and CNS neurones (Irving, Collingridge & Schofield, 1992; Llano, Dipolo & Marty, 1994). Despite its existence in nerve cells, however, there is as yet little direct evidence for a physiological role for CICR in neuronal function.

In the current work we describe the properties of a calcium transient induced by APs in acutely isolated nodose neurones and provide evidence that this calcium transient is produced by CICR.

METHODS

Cell dissociation

New Zealand White rabbits of either sex (1–2 kg body weight) were obtained from Robinson Services Inc. (NC, USA) and killed by sodium pentobarbitone overdose (100 mg kg^{-1}). Dissociated nodose neurones were prepared as previously described (Leal-Cardoso, Koschorke, Taylor & Weinreich, 1993) with the exception that a sterile technique was used and the final neuronal pellet was resuspended in sufficient Leibovitz L-15 medium (Gibco-BRL, NY, USA) containing 10% fetal bovine serum to plate 0·1 ml of cell suspension onto 25 mm circular glass coverslips (Fisher Scientific, NY, USA) coated with polylysine (0·1 mg ml^{-1} poly-D-lysine, Sigma). After a 3 h incubation at 37 °C, neurones were maintained at room temperature (22–24 °C) to minimize neurite outgrowth and were suitable for experimental use from 3 h to at least 3–4 days after dissociation (Magee & Schofield, 1991, and authors' own observations). Neither the sex of the animals nor the length of time neurones were kept in culture had any effect on the basic electrophysiological properties of the cells.

Electrode fabrication, recording chamber and drug delivery

Microelectrodes for intracellular recording were fabricated on a Flaming/Brown model P-87 micropipette puller (Sutter Instrument Co., CA, USA). The electrodes had DC resistances ranging from 30 to 70 MΩ when filled with 4 M potassium acetate.

A recording chamber was designed to provide superfusion of the 25 mm coverslip with physiological salt solution (for composition see below) via a gravity-flow system. The chamber was mounted on the stage of an inverted microscope (Diaphot, Nikon) equipped with a ×40 phase-contrast oil-immersion objective (Fluor, numerical aperture 1·3, Nikon) to allow direct visualization of neurones for intracellular impalement and for fluorescence measurements.

Electrophysiological recording

Standard intracellular stimulating and recording techniques were used to monitor electrical activity with intracellular glass micropipettes (see Christian, Taylor & Weinreich, 1989, for details). Current-clamp recordings were made with an Axoclamp-2A amplifier (Axon Instruments) either in bridge mode (filtering at 10 kHz) or in discontinuous mode (sample rate 5 kHz, filtered at 1 kHz). Current and voltage signals were stored on an instrumentation tape recorder (Racal Recorders Ltd) or on videocassette tapes via a Neurocorder (NeuroData Instruments, Inc., NY, USA) for off-line analysis. The membrane input resistance of the cell was monitored by measuring the magnitude of electrotonic voltage transients produced by 100 pA, 150 ms hyperpolarizing current pulses. Analysis of electrophysiological data was performed with pCLAMP 5.0 and 6.1 software (Axon Instruments). Neurones were accepted for study only if they showed a stable resting membrane potential (< -50 mV) throughout the experiment, an AP overshoot greater than 20 mV, and a membrane input resistance greater than 30 MΩ.

During experiments the cells were superfused (3–5 ml min^{-1}) with Locke solution (at 21–24 °C) which had the following composition (mM): 136 NaCl, 5·6 KCl, 1·2 NaH_2PO_4, 14·3 $NaHCO_3$, 1·2 $MgCl_2$, 2·2 $CaCl_2$ and 10·0 dextrose, equilibrated with 95% O_2–5% CO_2 and adjusted to pH 7·2–7·4 with NaOH.

To estimate the amount of Ca^{2+} entering a nodose neurone with each AP, we employed the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) and ionic conditions for isolating Ca^{2+} currents (Scroggs & Fox, 1992). Voltage-clamp experiments were performed with an Axopatch-1C amplifier (Axon Instruments) using pre-recorded nodose neurone APs as voltage-clamp commands (see Scroggs & Fox, 1992, and Fig. 4). The external solution contained (mM): 110 TEA-Cl, 2 $CaCl_2$, 10 Hepes and 100 nM TTX. The pipette solution contained (mM): 120 CsCl, 5 Na_2-ATP, 20 Hepes, 0·4 Tris-GTP and 10 EGTA. The pH of the external solution was adjusted with TEA-OH to 7·4 and the osmolality to 316 mosmol kg^{-1} with TEA-Cl. The pH of the internal solution was adjusted to 7·3 using CsOH and the osmolality to 303 mosmol kg^{-1} with CsCl.

Neurones were only admitted for whole-cell voltage-clamp study if their zero-current potential exceeded −60 mV, and the series resistance was stable and < 15 MΩ. Data were sampled at 40 kHz, filtered at 5 kHz, and stored as pCLAMP files for further analysis. The inward Ca^{2+} current (I_{Ca}) entering the neurone with each AP command was recorded, and the current trace was integrated to obtain the amount of charge moved across the membrane with each AP.

Reagents

Reagents were procured from the following vendors: caffeine from Sigma, thapsigargin from LC Laboratories (MA, USA), ryanodine from Calbiochem, fura-2 AM (the acetoxymethyl ester form) and Pluronic F-127 from Molecular Probes. Unless otherwise noted, drug solutions were prepared daily from concentrated ($\geqslant 10$ mM) stock solutions that were stored frozen at −20 °C. Drugs were delivered via the superfusate by switching a three-way valve to a reservoir containing a known concentration of the drug in oxygenated Locke solution. Where stock solutions in DMSO were diluted with aqueous media to give working solutions, DMSO concentration was $< 0{\cdot}2\%$ (v/v). When Pluronic F-127 was present in incubation media, its concentration was $< 0{\cdot}015\%$ (w/v). At these concentrations in normal Locke solution, neither DMSO nor Pluronic F-127 produced any discernible electrophysiological changes.

$[Ca^{2+}]_i$ measurement

To measure $[Ca^{2+}]_i$, neurones on coverslips were incubated for 40–60 min at room temperature (22–24 °C) in a solution containing 1 μM fura-2 AM as previously described (Cohen, Weinreich & Kao, 1994). After incubation the coverslip was placed in the recording chamber and superfused with Locke solution. Fura-2 fluorescence measurements were performed with a dual-excitation spectrofluorometer (CM1T10I, SPEX Industries, NJ, USA) coupled to the microscope through a fibre-optic cable and operating in the microfluorometry mode. Each neurone under study was alternately illuminated with 340 and 380 nm light, and the fluorescence emission, after passing through a 510 nm bandpass filter, was sampled by a photomultiplier tube, the output from which was digitized and stored for subsequent analysis. Instrument control,

data acquisition and analysis were performed through DM3000CM software (SPEX Industries) running on a dedicated PC.

$[Ca^{2+}]_i$ calibration

Values of $[Ca^{2+}]_i$ were derived using the ratio method of Grynkiewicz, Poenie & Tsien (1985). All fura-2 fluorescence records were corrected for background fluorescence by subtracting the light intensity measured from neurones depleted of fura-2 by digitonin permeabilization (Kao, 1994). $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz , Poenie & Tsien (1985):

$$[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)][S_{f2}/S_{b2}],$$

where R is the ratio F_{340}/F_{380}, R_{min} and R_{max} are the minimum and maximum values of the ratio, attained at zero and saturating Ca^{2+} concentrations, respectively. F_{340} is the fluorescence emitted by the dye when excited at 340 nm and F_{380} is the fluorescence emitted by the dye when excited at 380 nm. S_{f2}/S_{b2} is the ratio of fluorescence intensities for Ca^{2+}-free and Ca^{2+}-bound indicator measured with 380 nm excitation. Values of R_{min}, R_{max} and S_{f2}/S_{b2} were determined from six acutely dissociated neurones used specifically for calibration purposes.

Estimation of fura-2 concentration in nodose neurones

Dissociated neurones from single nodose ganglia were incubated, while being gently agitated on a tilt table, for 50–60 min at 22–24 °C in Hepes-buffered Leibovitz L-15 medium containing 1 μM fura-2 AM in codispersion with Pluronic F-127. The neurones (14 000–16 000 per sample) were pelleted by centrifugation (650 *g*, 5 min). The supernatant containing fura-2 AM was discarded and the cells were washed by resuspension and pelleting in Locke solution. The washed neurones were resuspended in 750–800 μl Locke solution and 1–2 μl of the cell suspension was used for cell counting and cell size measurements. The remaining neurones were incubated for 5 min at room temperature with digitonin at 20 μM, a concentration that is known to release low molecular weight cytosolic solutes but leaves organellar contents essentially intact (Kao, 1994). The digitonin lysate was separated from cell debris by centrifugation (10 000 *g*, 3 min). The pellet was solubilized by the addition of 700 μl Locke solution containing 1% Triton X-100 to release any fura-2 compartmentalized within organelles. The complete excitation spectra for the digitonin and Triton X-100 lysates were recorded. Corresponding spectra, recorded on control lysates from a replicate sample of neurones that had experienced identical treatment except that fura-2 AM was ommitted, were used to establish control baselines. Aliquots of standard fura-2 solution of known concentration were then added to the control digitonin and Triton X-100 lysates to generate standard spectra against which the spectra from fura-2 AM-loaded cell lysates could be compared quantitatively. By comparing the integrated fluorescence intensity from fura-2 AM-loaded lysates and control lysates containing known amounts of fura-2, it was possible to quantify the absolute amount of fura-2 in the cytosolic and organellar compartments of neurones incubated with fura-2 AM. Knowing the neuronal density and average neuronal volume from separate measurements, an estimate of the average concentration of dye in cytosolic and organellar compartments could be calculated. We thus estimate the mean cytosolic concentration of fura-2 in nodose neurones to be between 50 and 60 μM ($n = 2$ animals) after a 50–60 min incubation at 23 °C with 1 μM fura-2 AM.

Data are expressed as means ± S.E.M. ANOVA and Student's *t* test were used to assess significant differences between calculated means; $P < 0.05$ was considered significant. Unless specified, results reported were replicated in at least three neurones.

RESULTS

Resting $[Ca^{2+}]_i$

In acutely isolated adult rabbit nodose neurones loaded with fura-2, resting $[Ca^{2+}]_i$ averaged 80 ± 4·3 nM ($n = 19$, pooled data). In this sample, there was a statistical difference ($P < 0.05$) in the resting $[Ca^{2+}]_i$ for impaled *versus* non-impaled neurones (87 ± 3·6, $n = 13$ *versus* 61 ± 2·4 nM, $n = 6$, respectively).

Temporal characteristics and magnitude of Ca^{2+} transients

Transient increases in $[Ca^{2+}]_i$ (calcium transients) lasting several seconds could be evoked by APs generated by 1–5 ms depolarizing current pulses. The data in Figs 1 and 5 show the temporal characteristics of calcium transients produced by varying numbers of APs elicited at 10 Hz. The time to peak of calcium transients remained unchanged at 1·0 ± 0·06 s ($n = 32$) for one to eight APs. The decay of calcium transients always followed an exponential time course, with time (τ) values remaining unchanged for one to thirty-two APs (3·4 ± 0·29 s, $n = 39$).

Over the range of one to four APs, the amplitude of the calcium transient increased quasi-linearly with a slope of 9·6 ± 0·01 nM AP^{-1} ($r = 0.999$, $n = 10$; see Fig. 6). From neurone to neurone, there was considerable scatter in the magnitude of the calcium transients elicited by an AP (3·6–22·5 nM). This variability was not due to changes in the amount of Ca^{2+} entering nodose neurones with each AP because the time integral of the inward Ca^{2+} current (I_{Ca}) per AP does not change significantly over the range of one to thirty-two APs, nor does it vary significantly from cell to cell (see data below). Another possibility for the scatter might be the existence of cell-to-cell variability in the final intracellular fura-2 concentration. That is, higher levels of intracellular fura-2 loading corresponds to increased exogenous Ca^{2+} buffering capacity and thus might tend to diminish the measured amplitude of calcium transients. Although we determined the average intracellular fura-2 concentration in populations of nodose neurones to be 50–60 μM following a 50–60 min incubation with 1 μM fura-2 AM (see Methods), such population-averaged measurements provide no information on potential cell-to-cell variations in fura-2 loading. We examined potential effects of variable fura-2 loading by testing whether a correlation existed between the total fura-2 fluorescence signal from each neurone (the total output of the photomultiplier tube) and the magnitude of the calcium transient in twelve neurones. In these neurones where calcium transient per AP ranged between 4 and 20 nM, the net fluorescence signal was not correlated with the magnitude of the calcium transient ($r = 0.426$, $P = 0.167$, $n = 12$). The variability in the magnitude of the calcium transients, therefore is not the result of variable exogenous Ca^{2+} buffering arising from cell-to-cell differences in fura-2 loading. We suspect that the scatter in the magnitudes of

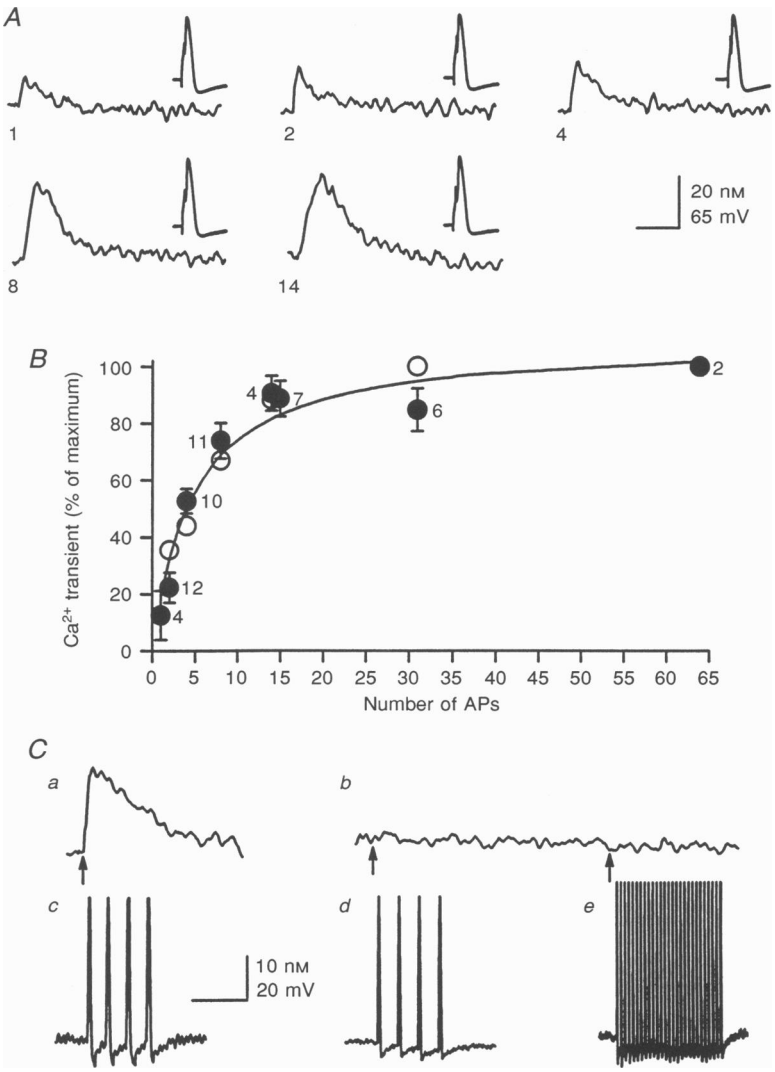

Figure 1. Effects of varying numbers of APs and nominally Ca^{2+}-free Locke solution on the amplitude of Ca^{2+} transients recorded in acutely isolated nodose neurones

A, sample traces are calcium transients evoked by different numbers of APs indicated below each trace. AP waveforms shown above the calcium transients depict the last AP in each train (10 Hz stimulation). The time calibration for APs and calcium transients is 20 ms and 3 s, respectively. *B*, the normalized amplitude of the calcium transients shown in *A* is plotted (○) against the number of APs. Also depicted is the mean calcium transient amplitude (± S.E.M.; ●) produced by varying numbers of APs recorded in several nodose neurones (number of neurones indicated near ●). The amplitude of each calcium transient is normalized to the maximal response observed in the same neurone. The continuous curve is a rectangular hyperbola fit to the data points ($\chi^2 = 3{\cdot}53$, $r = 0{\cdot}968$). *C*, trace *a* represents a calcium transient evoked by a train of 4 APs (shown in *c*) was recorded from a neurone that is being superfused with normal Locke solution ($[Ca^{2+}]_o = 2{\cdot}2$ mM). When the neurone was superfused with nominally Ca^{2+}-free Locke solution, no calcium transient could be evoked (*b*). APs shown in *d* and *e* were recorded 30 and 68 s after switching to nominally Ca^{2+}-free Locke solution, respectively. Even with a train of 27 APs (*e*), no calcium transient was observed. Lowering the $[Ca^{2+}]_o$ also resulted in a decrease in the size of the fast spike after-hyperpolarization (AHP_{fast}). The remaining components of the AHP_{fast} (traces *d* and *e*) probably reflect the delayed rectifier current. The time calibration is 10 s for *a* and *b*, 0·3 s for *c* and *d*, and 1·25 s for *e*. APs were generated by transmembrane depolarizing current pulses (5 nA, 1 ms, 10 Hz). Arrowheads mark the times when APs were evoked. Spike amplitudes shown in 1*C* are truncated. Resting membrane potential and input resistance were −54 mV and 50 MΩ, respectively. Fluorescence data were acquired at 10 Hz.

calcium transients reflects variability in release from internal stores of Ca^{2+}, perhaps mirroring the disparate functional properties of these visceral afferents (review by Mei, 1983).

Ca^{2+} sensitivity of Ca^{2+} transients

Calcium transients were sensitive to $[Ca^{2+}]_o$. Figure 1*C* shows a calcium transient induced by four APs. This calcium transient was completely abolished within 30 s of switching the superfusing Locke solution to one containing nominally zero Ca^{2+} (Fig. 1*Cb*). Similar results were observed in three additional neurones where lowering $[Ca^{2+}]_o$ resulted in rapid and complete abolition of the calcium transient. Increasing the number of spikes from four to twenty-seven failed to evoke a measurable calcium transient under these conditions (Fig. 1*Ce*). We monitored the effectiveness of reducing $[Ca^{2+}]_o$ by measuring changes in the magnitude of the fast spike after-hyperpolarization (AHP_{fast}). We have previously demonstrated that a major fraction of the AHP_{fast} is Ca^{2+} dependent (Fowler, Greene & Weinreich, 1985). The Ca^{2+} sensitivity of the AHP_{fast} was confirmed in these experiments by the reduced magnitude of the AHP_{fast} after switching to nominally Ca^{2+}-free Locke solution (compare Fig. 1*Cc* and *d*). The residual component of the AHP_{fast} in nominally Ca^{2+}-free Locke solution (Fig. 1*Cd*) is presumably mediated by the delayed rectifier. APs of shorter duration were observed in nominally Ca^{2+}-free Locke solution, probably reflecting the loss of a Ca^{2+} component of the AP. The omission of Ca^{2+} from the superfusing medium did not alter ($\leqslant 5\%$) other neuronal membrane properties, e.g. resting membrane potential and membrane input resistance.

Relation between APs and Ca^{2+} transients

When the number of APs was varied over the range one to sixty-five, the amplitude of the calcium transient increased rapidly at first and then began to level off with each succeeding AP and almost reached a plateau by the 65th AP. The relation between the amplitude of the calcium transient and number of APs was well fitted ($\chi^2 = 3{\cdot}53$) by a rectangular hyperbola (Fig. 1*B*). The traces shown in Fig. 1*A* illustrate the change in magnitude of the calcium transient recorded in one neurone, over the range of one to fourteen APs. Figure 1*B* shows the population results relating the normalized amplitude of the calcium transient with varying number of APs up to sixty-five. A similar hyperbolic relation was also observed if the integral of the calcium transient was plotted against number of APs (data

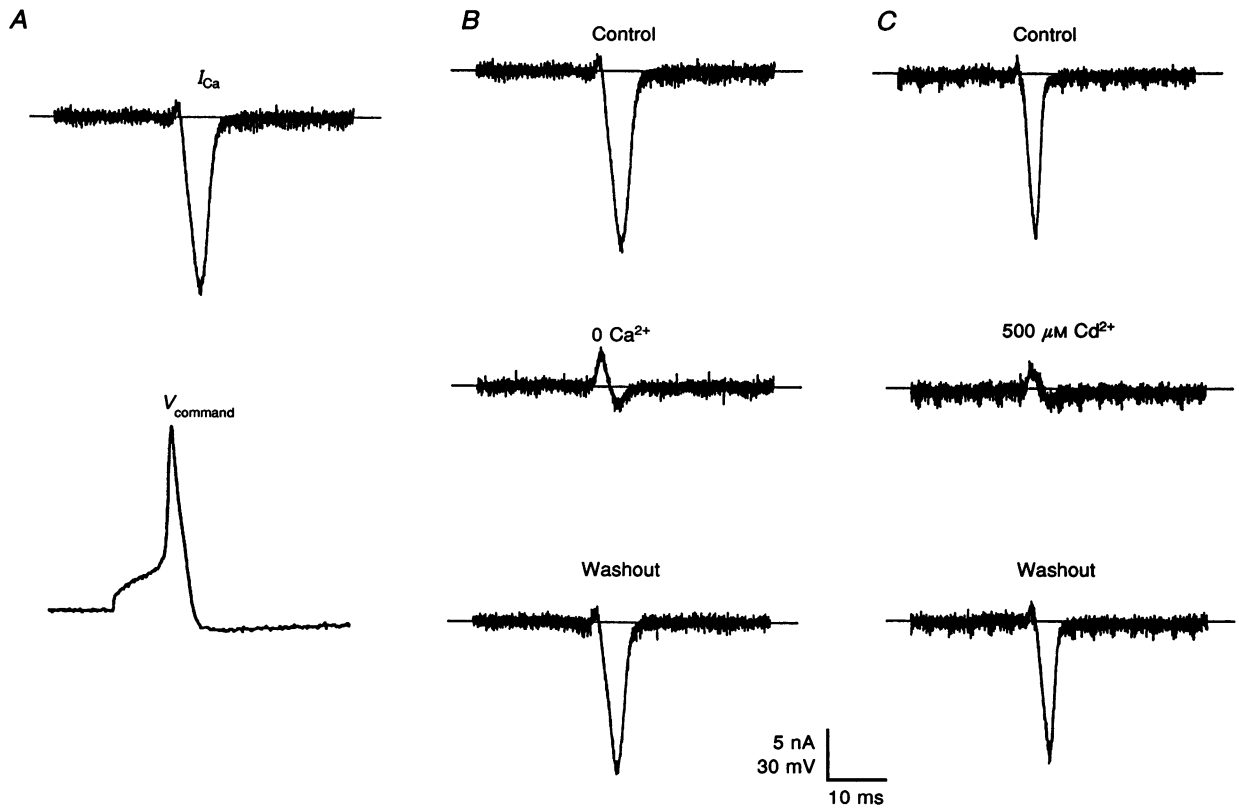

Figure 2. Calcium currents (I_{Ca}) elicited by nodose neurone AP waveforms

A, inward I_{Ca} (top trace) evoked by an AP voltage-clamp command from −60 to +50 mV (lower trace). *B*, effect of $[Ca^{2+}]_o$ on the amplitude of I_{Ca}. Switching from control to nominally zero $[Ca^{2+}]_o$ (middle trace) reduced I_{Ca} by $> 90\%$. *C*, effect of blocking voltage-dependent calcium channels with Cd^{2+}. In Cd^{2+}-containing external solution, I_{Ca} was reduced to about the same extent as in zero $[Ca^{2+}]_o$. In *B* and *C*, return to normal external solution (Washout) restored I_{Ca}. The nature of the small outward currents was not investigated. Records in *A* and *B* are from the same neurone. Horizontal lines on the I_{Ca} traces represent baseline currents. The holding potential was −60 mV.

Table 1. Effect of calcium modulators on charge movement corresponding to calcium influx (in pC) in acutely isolated adult rabbit nodose neurones

Condition	Control	Treatment	n
Zero $[Ca^{2+}]_o$	35 ± 9·8	2 ± 0·8	6
Cd^{2+} (500 μM)	27 ± 3·8	2 ± 0·4	7
DBHQ (10 μM)	30 ± 5·7	30 ± 4·6	3
Ryanodine (10 μM)	44 ± 6·9	43 ± 9·3	4
Thapsigargin (100 nM)	43 ± 5·3	35 ± 4·5	6

Calcium currents were evoked by AP voltage-clamp commands from −60 to +50 mV (see lower trace in Fig. 3*A* and Methods). Nodose neurones were voltage clamped with a patch pipette in the whole-cell configuration. Values shown are means ± S.E.M. of charge movement due to Ca^{2+} entering nodose neurones (in pC), determined by integrating I_{Ca}. For each condition, control I_{Ca} values were recorded first. The neurones were then incubated for various time periods with modified extracellular solutions and I_{Ca} values were re-recorded. n, number of neurones used for each condition. Neurones were incubated in extracellular solution that contained nominally zero Ca^{2+} (for 3–10 min) or Cd^{2+} (for 3–8 min), or the reagents 2,5-di(*t*-butyl)hydroquinone (DBHQ), ryanodine, or thapsigargin (for 5–7 min), before I_{Ca} values were determined. DBHQ, ryanodine, or thapsigargin did not significantly depress the amount of Ca^{2+} influx ($P < 0·05$).

not shown). The voltage traces shown above the calcium transients in Fig. 1*A* are the last AP waveforms in the trains used to elicit the calcium transient. Over the range of one to sixteen APs elicited by 10 Hz depolarizing current pulses, the spike duration (at half peak amplitude and at baseline), peak overshoot and amplitude of the AHP_{fast} did not change significantly ($P = 0·993$, 0·951 and 0·998, respectively; $n = 6$).

There are several possible interpretations for the hyperbolic relation between the magnitude of the calcium transient and the number of APs. First, the levelling of the slope could reflect indicator saturation. This is highly unlikely, because bath application of ionomycin (10 μM) or digitonin (20 μM) to permeabilize the neurone always resulted in a marked increase in the fura-2 F_{340}/F_{380} ratio (data not shown), suggesting that the indicator was not near saturation. A second possibility is that with large numbers of APs the elevation in $[Ca^{2+}]_i$ might be sufficient to activate mitochondrial sequestration of Ca^{2+}, which would tend to suppress Ca^{2+} transients once they rise above the mitochondrial 'setpoint' (micromolar concentrations; Miller, 1987). This possibility also seems unlikely because $[Ca^{2+}]_i$ never reached levels approaching the mitochondrial setpoint in our experiments. A third possibility is that the amount of Ca^{2+} entering through VDCCs with each AP may not be constant and may decrease with each successive AP in the train. This, too, seems unlikely because the AP waveform did not change during the train. Nonetheless, the experiments described below address this concern in greater detail.

Does the magnitude of Ca^{2+} influx produced by an AP change during a spike train?

To establish the amount of Ca^{2+} entering a neurone with each AP we used a procedure similar to that described by Scroggs & Fox (1992); namely, recording whole-cell currents evoked by a pre-recorded AP used as a voltage-clamp command. Under these experimental conditions the major inward charge carrier is Ca^{2+} (see Methods). The upper trace in Fig. 2*A* shows a Ca^{2+} current (I_{Ca}) elicited by a nodose neurone AP voltage command, shown in the lower trace of Fig. 2*A*. To verify that the inward current was indeed mediated by Ca^{2+} influx, inward currents were evoked in normal and in nominally Ca^{2+}-free external solution. In nominally Ca^{2+}-free solutions, voltage-clamp commands produced only small inward currents (< 7% of control, Fig. 2*B* middle trace and Table 1). Upon reintroduction of normal external solution, I_{Ca} returned to near control amplitude (lower trace, Fig. 2*B*). Addition of 500 μM Cd^{2+} to normal external solution also decreased the amplitude of I_{Ca} to less than 8% of control (Fig. 2*C* and Table 1). A small outward current was usually present in the records of I_{Ca} (see traces in Figs 2, 3 and 7). The nature of these outward currents was not further investigated; however, we suspect they may reflect current flow through non-specific cation channels. By integrating I_{Ca}, we calculated that the amount of electrical charge due to Ca^{2+} entry resulting from a single AP was 39 ± 2·8 pC ($n = 30$).

The data in Fig. 3 address the question whether the magnitude of I_{Ca} changes during a spike train. Voltage-clamp commands consisting of one to thirty-two pre-recorded APs, delivered at 10 Hz, were applied to nodose neurones while I_{Ca} was monitored. Each current trace in Fig. 3 is a superposition of all the individual records of I_{Ca} evoked by a given train of voltage-clamp commands. The amplitude and the integral of I_{Ca} evoked by each AP did not change significantly with increasing numbers of APs, over the range of one to thirty-two spikes. These data therefore indicate that the plateau observed in the relation between the magnitude of calcium transients and the number of APs (see Fig. 1) cannot be explained by a diminution in spike-induced Ca^{2+} influx. A more probable explanation is that the

Table 2. Effect of reagents that modulate CICR on the magnitude of caffeine-induced Ca^{2+} transients in acutely isolated rabbit nodose neurones

Condition	Peak 1 (%)	Peak 2 (%)	Peak 3 (%)	n
Caffeine (10 mM)	100	75 ± 20·7	85 ± 12·6	5
Zero $[Ca^{2+}]_o$	6 ± 0·6	1 ± 1·7	0	3
Thapsigargin (100 nM)	6 ± 7·0	6 ± 7·7	5 ± 6·5	4
Ryanodine (10 μM)	0	0	0	6
DBHQ (10 μM)	8 ± 8·2	3 ± 3·0	—	5

The integral of the $[Ca^{2+}]_i$ trace was measured during 2 min bath applications of caffeine (10 mM) delivered at 3 min intervals. Because the first caffeine response was always larger than the 2 subsequent responses, repetitive caffeine responses were normalized to the magnitude of the first caffeine-induced calcium transient. The changes in the caffeine response in the presence of various reagents or in lowered $[Ca^{2+}]_o$ are calculated as percentage change normalized to the integral of the $[Ca^{2+}]_i$ trace of the 1st, 2nd or 3rd control caffeine response, respectively. Locke solution containing thapsigargin, ryanodine, DBHQ or nominally zero $[Ca^{2+}]_o$ was applied to the neurones for 4–31 min. Fluorescence data were acquired at 1 Hz. Data are expressed as means ± S.E.M.

decrease in calcium transient reflects a diminution in release from an intracellular Ca^{2+} store whose content is used to amplify the Ca^{2+} signal due to Ca^{2+} influx evoked by APs. A prime candidate for this store is the Ca^{2+}-induced Ca^{2+} release (CICR) pool that is well characterized in skeletal and cardiac muscle (for review see Wier, 1990) and has recently been documented in dorsal root ganglion neurones (Shmigol *et al.* 1995), in neurones in rabbit otic ganglia (Yoshizaki *et al.* 1995), as well as in other peripheral neurones (Kuba 1994).

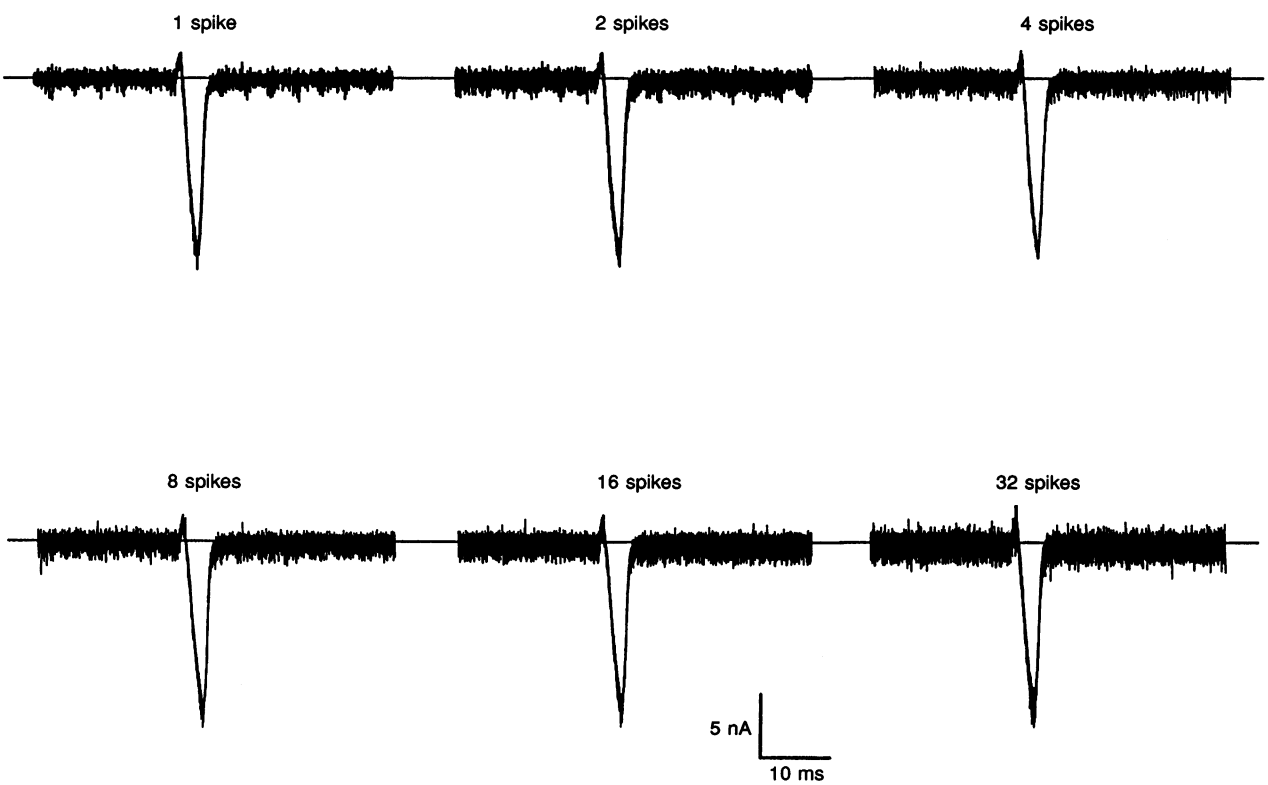

Figure 3. Effect of varying the number of AP waveforms on the magnitude of individual calcium currents (I_{Ca}) elicited by each AP

Each panel shows a superposition of all individual I_{Ca} traces evoked by a train of AP voltage-clamp commands. The number of AP waveforms within each train is indicated above each panel. Baseline current at holding potential (−60 mV) is represented by the horizontal lines through the traces. All traces recorded from the same neurone. There was no significant difference ($P < 0·05$) in the amplitudes or the integrals of the I_{Ca} produced by up to 32 APs.

Ca^{2+}-induced Ca^{2+} release in acutely isolated nodose neurones

The inference that CICR contributes to calcium transients depends upon several criteria (Endo, 1975; Lipscombe *et al.* 1988, Llano *et al.* 1994). First, because CICR is triggered by Ca^{2+} entry due to an AP, calcium transients should be abolished when external Ca^{2+} is removed. Second, the rise time of the calcium transient must outlast the duration of I_{Ca}, and third, the calcium transient should be altered by agonists and antagonists known to modulate CICR.

We have shown that the calcium transient is abolished when APs are elicited in nominally Ca^{2+}-free Locke solution (Fig. 1), that it is blocked by intracellular BAPTA (Cohen *et al.* 1994), and that it has a protracted time to peak ($1{\cdot}0 \pm 0{\cdot}06$ s for 1–8 APs, $n = 32$). The time to peak of the calcium transient is substantially longer than the 7–10 ms duration for the I_{Ca} induced by an AP voltage-clamp command (see Figs 2, 3 and 7). These characteristics suggest that the calcium transients are probably associated with CICR. Below we provide pharmacological evidence that spike-induced calcium transients are produced by CICR.

Superfusing neurones with Locke solution containing 10 mM caffeine, a known agonist of CICR (Endo, 1975), consistently produced a rise in $[Ca^{2+}]_i$ (25 of 25 cells; see Fig. 4*A* and *C*). When neurones were superfused with nominally Ca^{2+}-free Locke solution, the response to caffeine was rapidly attenuated and then completely abolished (Fig. 4*B* and Table 2).

When neurones were superfused with Locke solution containing 10 μM ryanodine, an antagonist of CICR (Fill & Coronado, 1988), subsequent superfusion with 10 mM caffeine no longer induced a measurable calcium transient (Fig. 4*D* and Table 2). We also assessed the ability of thapsigargin and 2,5-di(*t*-butyl)hydroquinone (DBHQ), two Ca^{2+}-ATPase inhibitors that functionally inhibit CICR, to modify caffeine-induced calcium transients (reviewed by Inesi & Sagara, 1994). Each reagent strongly attenuated ($\geqslant 93\%$) the caffeine-induced calcium transients (Table 2). These pharmacological results indicate that caffeine is acting to release Ca^{2+} from CICR stores in nodose neurones.

AP-induced CICR

Calcium transients can be evoked by single APs in control nodose neurones (see Figs 1 and 6). In contrast, after neurones were treated with ryanodine, thapsigargin or DBHQ at concentrations and for durations that were previously shown to block caffeine-induced calcium transients (Table 2), at least eight APs were required before a measurable calcium transient could be observed. The data in Fig. 5*B* illustrate the relation between the magnitude of the calcium transient and number of APs in three neurones

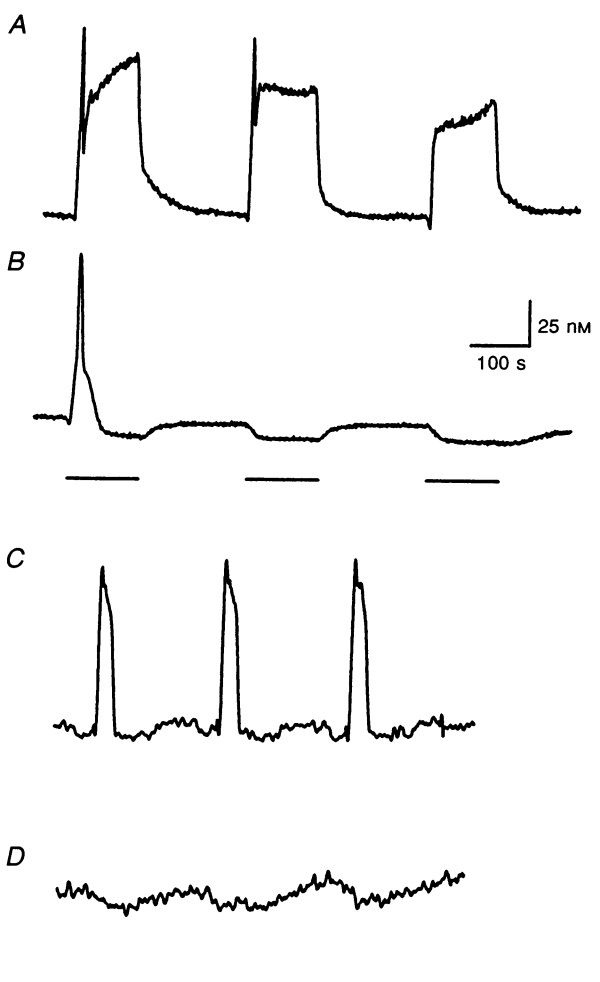

Figure 4. Effects of low extracellular Ca^{2+} and ryanodine on caffeine-induced Ca^{2+} transients recorded in isolated rabbit nodose neurones

A, calcium transients produced by repeated applications of 10 mM caffeine. The spike-like response on the rising phase of the caffeine-induced calcium transient was not always observed. *B*, caffeine-induced calcium transients became truncated and were eventually completely abolished when the neurone was superfused with nominally Ca^{2+}-free Locke solution. The first response to caffeine was recorded 65 s after switching to nominally Ca^{2+}-free Locke solution. *C*, control caffeine-induced calcium transients recorded in another neurone. The more abbreviated caffeine-induced calcium transients shown in this neurone (compare with calcium transients shown in *A*) was observed in about 40% of the neurones. *D*, application of 10 μM ryanodine, beginning 8 min before caffeine application, abolished the caffeine-induced calcium transient in this neurone. The horizontal bars below traces *B* and *D* represent the duration of caffeine exposure. A summary of these experiments is shown in Table 2. The caffeine-induced decrease in fluorescence observed in traces *B* and *D* is due to an interaction between the caffeine and fura-2 which enhanced F_{380} more than F_{340} and thus artifactually leads to a diminished F_{340}/F_{380} ratio (see Friel & Tsien, 1992 for details). Data were not corrected for this artifact. Fluorescence data were acquired at 1 Hz.

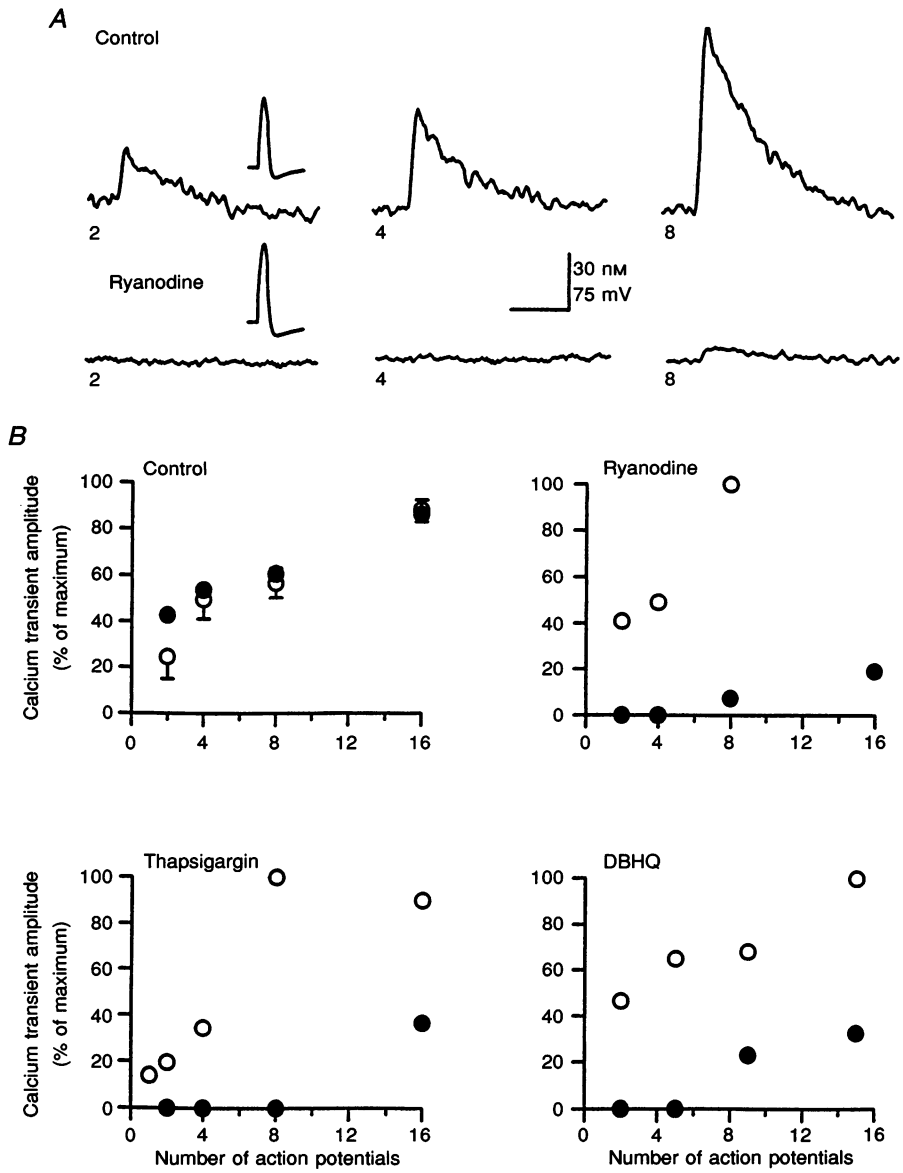

Figure 5. Effects of ryanodine and Ca^{2+}-ATPase inhibitors on the relation between the amplitude of the spike-induced Ca^{2+} transients and the number of APs

A, calcium transients evoked by varying number of APs (indicated below each trace) in control Locke solution and after 15 min incubation in a Locke solution containing 10 μM ryanodine. The APs depicted above the calcium transients are the 2nd waveform in a train elicited at 10 Hz. There was no significant effect of ryanodine on the amplitude or duration of the spike, or the magnitude of the fast post-spike after-hyperpolarization (see text). Time calibration for APs and for calcium transients is 20 ms and 3 s, respectively. Fluorescence data were acquired at 10 Hz. The relationship between the amplitude of these calcium transients and the number of APs is depicted in the plot labelled Ryanodine in *B*. The effects of incubating two additional nodose neurones in Ca^{2+}-ATPase inhibitors, thapsigargin (100 nM, for 20 min) or DBHQ (10 μM, for 8 min), are also illustrated. For each neurone, all calcium transients are normalized to the maximal calcium transient amplitude recorded in the same cell in control Locke solution. The plot labelled Control in *B* examines whether time of incubation affects the relation between the amplitude of calcium transients and the number of APs. Data from each of four nodose neurones are normalized to the maximum response recorded in that neurone. Incubation in drug-free Locke solution for 10–20 min did not alter the calcium transient–AP relation. Symbols are mean ± S.E.M. values at zero time (○) and after 10–20 min of incubation (●). In the Control panel, where not shown, error bars are within symbols. APs were generated by transmembrane depolarizing current pulses (1·5 nA, 5 ms, 10 Hz).

before and after exposure to 10 μM ryanodine, 100 nM thapsigargin or 10 μM DBHQ. The control data points (○) show the increase in the magnitude of the calcium transient as the number of APs was increased over the range of one to sixteen APs. However, following treatment with CICR inhibitors, no calcium transient could be observed with fewer than eight APs. Figure 5*A* depicts sample calcium transient traces elicited by two, four and eight APs in control Locke solution and in Locke solution containing 10 μM ryanodine. Comparisons of the amplitudes of the eight-spike calcium transient reveals that ryanodine treatment reduced the calcium transient more than 10-fold. This diminution in the magnitude of the calcium transient may underestimate the true changes produced by CICR blockers or Ca^{2+}-ATPase inhibitors, because an eight-spike calcium transient elicited in normal Locke solution does not fall on the steeply rising, quasi-linear portion of the calcium transient–AP curve, where the contribution of CICR is the strongest (see Figs 1 and 5). We can estimate the contribution of AP-induced Ca^{2+} influx alone to changes in $[Ca^{2+}]_i$ by examining the calcium transient–AP relation obtained when CICR is blocked by inhibitors. Over the range of eight to thirty-one APs, the data were analysed by linear regression (Fig. 6). The slopes of the lines were 0·5 ± 0·23, 1·1 ± 0·02 and 0·8 ± 0·33 nM AP^{-1} for ryanodine-, DBHQ- and thapsigargin-treated neurones, respectively ($n = 3$, 5 and 3, respectively). When these slopes are compared with the control slope of 9·6 nM AP^{-1} derived above from the steep, quasi-linear portion of the calcium transient–AP curve observed for untreated cells (Fig. 6), it is evident that a substantial (approximately 10- to 20-fold) amplification of the calcium transient occurs when the CICR pool is unimpaired.

The above interpretation assumes that Ca^{2+} extrusion mechanisms do not affect the magnitude of the calcium transient. However, it is possible that at the end of long trains of APs, Ca^{2+} extrusion processes may already be under way. Such extrusion processes would make calcium transients elicited by long spike trains appear smaller than they would be otherwise. In consequence, linear fits of the type described above could give slopes that are lower than they might be in the absence of extrusion. A more conservative estimate of the slopes obtained in the presence of CICR inhibitors can be obtained by excluding calcium transients elicited by thirty-one APs from the linear fits. The slopes so obtained are 1·2, 1·1 and 1·9 nM AP^{-1}, for ryanodine-, DBHQ- and thapsigargin-treated neurones, respectively (e.g. dashed line, Fig. 6). Again, comparing these values with the control slope of 9·6 nM AP^{-1} reveals that CICR contributes an approximately 5- to 10-fold amplification. Thus, both analytical approaches lead to the conclusion that when the CICR pool is intact, it is capable of substantially amplifying the Ca^{2+} signal arising from AP-induced Ca^{2+} influx.

The data acquired when CICR is abolished by pharmacological manipulations also provides some insight into the Ca^{2+} buffering capacity of rabbit nodose neurones. Under these conditions at least eight APs were needed to cause any detectable calcium transient. This suggests that the endogenous Ca^{2+} buffers in these neurones are robust and that they can buffer the influx of Ca^{2+} produced by at least four and possibly up to seven APs.

We attempted to validate several assumptions inherent to the use of CICR antagonists. For example, to achieve a 90–100% inhibition of CICR, as measured by a block of caffeine-induced calcium transients (see Table 2), neurones were incubated for varying time periods with CICR blockers. Because it is conceivable, though improbable, that incubation *per se* could modify the calcium transient–AP relation, we examined whether incubation time could alter this relation. The 'Control' panel in Fig. 5*B* summarizes the results from four neurones where the calcium transient–AP relation was determined and then re-determined after incubation in normal Locke solution for 10–20 min. The data show that incubation times up to 20 min do not produce significant changes in the relation between the

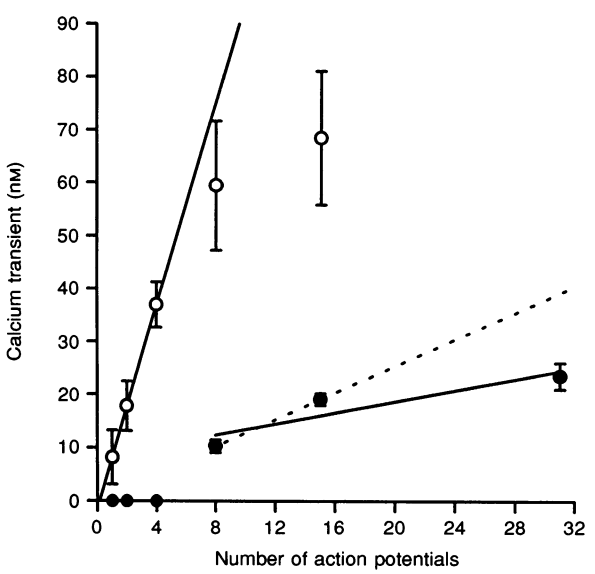

Figure 6. Effects of ryanodine on the relation between the amplitude of Ca^{2+} transients and the number of APs

Values are calcium transient amplitudes evoked by varying number of APs for control ($n = 10$; ○) and for ryanodine-treated ($n = 3$; ●) nodose neurones. Where not shown, error bars are within symbols. No calcium transient was observed with fewer than 8 APs in ryanodine-treated neurones (see also Fig. 5). Linear regression of data from control cells yielded a slope of 9·6 ± 0·01 nM AP^{-1} (1–4 spikes; $r = 0·999$). For ryanodine-treated cells, the slope was 0·5 ± 0·23 nM AP^{-1}, when data for 8–31 spikes were analysed (continuous line, $r = 0·916$). At the end of long trains of APs (e.g. 31 spikes), Ca^{2+} extrusion mechanisms may reduce the calcium transient amplitude. Therefore, a fit was also performed where the response to 31 APs was excluded (dashed line), yielding a slope of 1·2 nM AP^{-1}.

magnitude of calcium transients and the number of APs over the range of two to sixteen APs.

Several drugs that interfere with the release of Ca^{2+} from intracellular stores have been reported to decrease calcium channel activity (Nelson *et al.* 1994). Therefore, it is possible that some of the changes in the spike-induced calcium transients observed after treatment with blockers of CICR or Ca^{2+}-ATPases could be attributed to a drug-induced reduction of Ca^{2+} influx. To examine this possibility, we incubated neurones for 5–7 min with 10 μM ryanodine, 10 μM DBHQ or 100 nM thapsigargin and examined whether these drugs altered the amplitude of I_{Ca} evoked by AP voltage-clamp commands. As shown in Fig. 7, and summarized in Table 1, ryanodine, DBHQ, or thapsigargin did not produce significant changes in the magnitude of I_{Ca} ($P = 0{\cdot}900$, $0{\cdot}930$ and $0{\cdot}210$, respectively). Finally, we examined whether ryanodine treatment affected the AP waveform. In five neurons subjected to 10 μM ryanodine for 5–20 min, the spike duration (at half peak amplitude or at baseline), peak overshoot and amplitude of the AHP_{fast} were not significantly different from control values ($P = 0{\cdot}420$, $0{\cdot}766$ and $0{\cdot}954$, respectively). Figure 5*A* shows representative AP waveforms recorded before and after a 15 min incubation in 10 μM ryanodine. Thapsigargin also produced no significant changes in the AP waveform ($P = 0{\cdot}718$, $0{\cdot}413$ and $0{\cdot}358$; $n = 3$).

DISCUSSION

Our principal finding is that nodose neurones have a process for amplifying intracellular Ca^{2+} signals following an AP. Without this process, essentially all of the Ca^{2+} influx produced by one to seven APs would be completely buffered by endogenous Ca^{2+} binding proteins and other Ca^{2+} sequestration mechanisms. In the aggregate, our data suggest that the process for amplification of the Ca^{2+} influx signal is CICR.

The CICR pool is one of several intracellular Ca^{2+} pools believed to be present in neurones (Kuba, 1994). In the present case, the conclusion that CICR exists in nodose

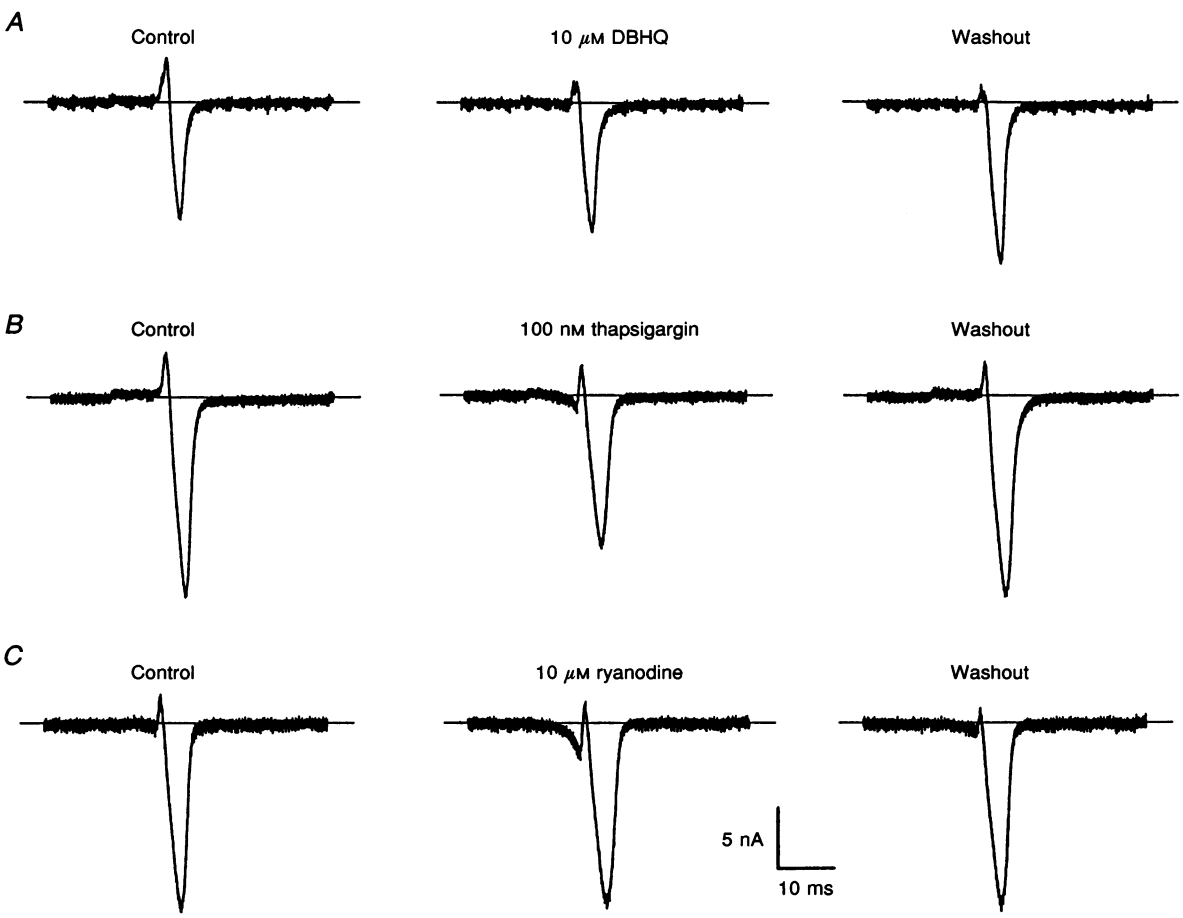

Figure 7. Effects of the CICR inhibitor ryanodine and Ca^{2+}-ATPase inhibitors thapsigargin and DBHQ on the magnitude of calcium currents

Calcium currents evoked by AP voltage-clamp commands recorded in three separate nodose neurones. Incubation times for *A–C* were 5–7 min. Baseline current at the holding potential (−60 mV) is represented by the horizontal line through the traces. DBHQ and ryanodine did not depress the magnitude of I_{Ca}, but in this neurone thapsigargin reduced I_{Ca} amplitude by ~15%. A summary of these experiments is shown in Table 1.

neurones is based on several types of observations of AP-induced calcium transients. First, calcium transients had a long time to peak, approximately 1 s, a time period that is orders of magnitude longer than the duration of the Ca^{2+} influx associated with an AP (Figs 2, 3 and 7). Although the time to peak of the calcium transients was clearly longer than the *ca* 10 ms associated with Ca^{2+} influx, we have not assessed whether the equilibration of Ca^{2+} within the neurones contributes significantly to the time to peak of the calcium transients. Second, caffeine, a well-characterized agonist of CICR, produced Ca^{2+} transients the magnitude of which was dependent upon $[Ca^{2+}]_o$ (Fig. 4). Third, ryanodine, DBHQ and thapsigargin abolished calcium transients without affecting AP-induced Ca^{2+} influx (Fig. 7 and Table 1), consistent with the CICR pool being the major source of Ca^{2+} for calcium transients in nodose neurones. Finally, at the same concentrations and incubation times that effectively blocked AP-induced calcium transients, these reagents abolished caffeine-induced Ca^{2+} transients in nodose neurones (Fig. 4 and Table 2). We thus conclude that CICR exists in rabbit nodose neurones.

CICR has been shown to have a Ca^{2+} activation threshold in cerebellar Purkinje neurones (Llano *et al.* 1994), in sympathetic ganglia neurones (Hua *et al.* 1993) and in dorsal root ganglion neurones (Shmigol *et al.* 1995). If a Ca^{2+} activation threshold exists in nodose neurones we would expect the relationship between the magnitude of calcium transients and the number of APs to be linear up to some number of APs. Beyond this point (the threshold), there would be an increase in the slope, signalling the appearance of an augmentation process that amplifies the effect of each additional AP. Such a slope shift would be interpreted as evidence for a Ca^{2+} activation threshold for CICR. However, when we plotted the magnitude (amplitude or integral) of calcium transients against the number of APs, the relation was always fitted by a rectangular hyperbola (Fig. 1). Several considerations indicate that it may be difficult to demonstrate a threshold for CICR in these neurones using APs as a trigger for Ca^{2+} influx. To observe calcium transients that are independent of CICR would require reducing the magnitude of the Ca^{2+} influx below that required to activate Ca^{2+} release from the CICR pool. However, because the magnitude of a single AP-induced calcium transient is already close to our limit of detection in normal Locke solution, it will be difficult to detect calcium transients under conditions where Ca^{2+} influx is reduced. A further complicating factor is that the size of the CICR pool in these neurones rapidly equilibrates to new levels, within 60 s, upon changing the concentration of extracellular Ca^{2+} (data not shown). Thus, finding conditions that may yield a bilinear calcium transient–AP relation may require the use of a more sensitive Ca^{2+} indicator and techniques that allow rapid alterations in the magnitude of the cytosolic Ca^{2+} load.

The absence of an obvious threshold for the induction of AP-induced calcium transients supports the view that CICR in nodose neurones can be activated even with a single AP. Indeed, the fact that a calcium transient resulting from a single AP is detected suggests a physiological role for CICR in these neurones.

An estimate of the contribution of CICR to the spike-induced calcium transient can be derived by comparing the slope of the control calcium transient–AP curve with the slopes of the calcium transient–AP relations observed in the presence of CICR inhibitors (Fig. 6). The slope of the control curve was 9·6 nM AP^{-1}, between one and four APs. The slopes for ryanodine-, DBHQ-, or thapsigargin-treated cells were 0·5, 1·1 and 0·8 nM AP^{-1}, respectively (8–31 spikes) or, more conservatively (see Results), 1·2, 1·1 and 1·9 nM AP^{-1}, respectively (8–16 spikes). Thus, in adult rabbit nodose neurones, CICR amplifies the increase in $[Ca^{2+}]_i$ due to AP-induced Ca^{2+} influx by at least 5- to 10-fold, and possibly up to 20-fold. In contrast, single-spike calcium transients were not observed in dorsal root ganglion (DRG) neurones from 2- to 7-day-old rats (Shmigol *et al.* 1995). However, with trains of 10–100 spikes, a linear calcium transient–AP relation could be constructed with a slope of ~3·5 nM AP^{-1}. With ryanodine treatment this slope remained unchanged up to fifty spikes (at 20 Hz), beyond which the slope decreased to ~1·5 nM AP^{-1}. Thus although CICR stores exist in DRG neurones, large Ca^{2+} loads appear necessary to trigger its release. In this regard it is interesting to note that for nodose and DRG neurones of comparable diameter, a single AP in nodose neurones causes a smaller influx of Ca^{2+} (39 *vs.* 49 pC, respectively; cf. Fig. 2 and Scroggs & Fox, 1992) yet this influx of Ca^{2+} is sufficient to trigger CICR in nodose but not in DRG neurones.

From the calcium transient–AP relation (Fig. 1), it appears that CICR is nearly maximally activated by the Ca^{2+} influx associated with about twenty APs. The fact that CICR can be activated with one AP and saturates with about twenty APs can operationally define a range where CICR amplification of the AP-induced Ca^{2+} influx signal is optimal. In the present experiments, different numbers of APs were evoked, but always at a frequency of 10 Hz. We do not yet know whether this or other frequencies of stimulation are optimal for amplifying the Ca^{2+} signal through CICR. If the time to peak of calcium transients (~1 s) were to constrain the 'best' frequency, then 1 Hz might actually be optimal. It is interesting to note that under resting conditions *in vivo*, afferent vagal C fibres innervating the airway have discharge rates of about 0·5–0·8 Hz and that this frequency increases to 3–20 Hz following various physiological stimuli (Coleridge & Coleridge, 1984).

Physiological role for CICR

The function of CICR in neurones has been the subject of many investigations but a specific physiological role for CICR in normal neuronal function remains elusive. Friel & Tsien (1992) first proposed that a CICR store in bullfrog sympathetic neurones might serve a dual purpose: acting as

either an intracellular Ca^{2+} sink or an intracellular Ca^{2+} source. The operational modality of this CICR store would be controlled by its Ca^{2+} content at any given time. If the store is low or empty, it could function as a sink, attenuating incoming Ca^{2+} signals; conversely, if the store is full it could function as a source, augmenting incoming Ca^{2+} signals.

Nodose neurones, on the other hand, appear to use CICR solely as an amplification process to raise $[Ca^{2+}]_i$. The elevation of $[Ca^{2+}]_i$ by CICR activation would thus be ideally suited to provide intracellular Ca^{2+} for any Ca^{2+}-dependent processes, particularly those with relatively slow onset kinetics. The CICR-dependent calcium transient produced by one AP lasts many seconds before returning to baseline. The slow time course of calcium transient decay could allow a 'residual' Ca^{2+} load (Katz & Miledi, 1967) for supporting spike frequency-dependent plasticity of any Ca^{2+}-dependent process. Experimental data for such a mechanism has recently been reported for dorsal root ganglion neurones (Lüscher, Lipp, Lüscher & Niggli, 1996), where the intracellular accumulation of Ca^{2+} observed during repetitive nerve stimulation can control AP propagation and safety factor, presumably because cumulatively elevated $[Ca^{2+}]_i$ caused Ca^{2+} channel inactivation.

Another role for Ca^{2+} released from CICR stores in nodose neurones might be to activate cytosolic Ca^{2+} binding proteins that mediate physiological changes. An interesting candidate protein for this purpose is the ubiquitous Ca^{2+}-binding regulatory protein calmodulin (Colbran, 1992). Calmodulin, when Ca^{2+} bound, can regulate many cellular processes, one of which is the activation of nitric oxide synthase (NOS) to produce nitric oxide (NO), a recently characterized biological messenger (Dawson & Snyder, 1994). NO generated by NOS has been shown to be important for the development of an AP-induced slow post-spike after-hyperpolarization (AHP_{slow}) that controls spike frequency adaptation in many nodose neurones (Weinreich & Wonderlin, 1987; Cohen *et al.* 1994). Thus, CICR may control excitability in nodose neurones by supporting NO synthesis. That CICR may be fundamental to the generation of AHP_{slow} has been inferred from the parallel time course of the calcium transient and the AHP_{slow} (Tatsumi, Hirai & Katayama, 1988; Cohen *et al.* 1994) and from numerous studies employing reagents that interfere with the release of Ca^{2+} from internal stores (see review by Sah, 1996). It remains to be demonstrated, however, that an AHP_{slow}, or its current, and the calcium transient are simultaneously blocked by pharmacological manipulations that selectively interfere with CICR.

In conclusion, CICR is present in rabbit nodose neurones and it can be activated by physiological stimuli. We suggest that a wide variety of Ca^{2+}-dependent processes within these neurones are regulated by this Ca^{2+} pool.

Berridge, M. J. & Irvine, R. F. (1989). Inositol phosphates and cell signalling. *Nature* **341**, 197–205.

Cheek, T. R. (1989). Spatial aspects of calcium signaling. *Journal of Cell Science* **93**, 211–216.

Christian, E. P., Taylor, G. E. & Weinreich, D. (1989). Serotonin increases excitability of rabbit C-fiber neurons by two distinct mechanisms. *Journal of Applied Physiology* **67**, 584–591.

Cohen, A. S., Weinreich, D. & Kao, J. P. Y. (1994). Nitric oxide regulates spike frequency accommodation in nodose neurons of the rabbit. *Neuroscience Letters* **173**, 17–20.

Colbran, R. J. (1992). Regulation and role of brain calcium/calmodulin-dependent protein kinase II. *Neurochemistry International* **21**, 469–497.

Coleridge, J. C. G. & Coleridge, H. M. (1984). Afferent vagal C fibre innervation of the lungs and airways and its functional significance. *Reviews of Physiology Biochemistry and Pharmacology* **99**, 1–110.

Dawson, T. M. & Snyder, S. H. (1994). Gases as biological messengers: Nitric oxide and carbon monoxide in the brain. *Journal of Neuroscience* **14**, 5147–5159.

Endo, M. (1975). Conditions required for calcium-induced release of calcium from the sarcoplasmic reticulum. *Proceedings of the Japan Academy* **51**, 467–472.

Endo, M. (1985). Calcium release from the sarcoplasmic reticulum. *Current Topics in Membranes and Transport* **25**, 183–230.

Fabiato, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology* **245**, C1–14.

Fill, M. & Coronado, R. (1988). Ryanodine receptor channel of sarcoplasmic reticulum. *Trends in Neurosciences* **11**, 453–457.

Fowler, J. C., Greene, R. & Weinreich, D. (1985). Two calcium-sensitive spike after-hyperpolarizations in visceral sensory neurones of the rabbit. *Journal of Physiology* **65**, 59–75.

Friel, D. D. & Tsien, R. W. (1992). A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurones modulates effects of Ca^{2+} entry on $[Ca^{2+}]_i$. *Journal of Physiology* **450**, 217–246.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Hua, S. Y., Nohmi, M. & Kuba, K. (1993). Characteristics of Ca^{2+} release induced by Ca^{2+} influx in cultured bullfrog sympathetic neurones. *Journal of Physiology* **464**, 245–272.

Inesi, G. & Sagara, Y. (1994). Specific inhibitors of intracellular Ca^{2+} transport ATPases. *Journal of Membrane Biology* **141**, 1–6.

Irving, A. J., Collingridge, G. L. & Schofield, J. G. (1992). Interactions between Ca^{2+} mobilizing mechanisms in cultured rat cerebellar granule cells. *Journal of Physiology* **456**, 667–680.

Kao, J. P. Y. (1994). Practical aspects of measuring $[Ca^{2+}]$ with fluorescent indicators. In *Methods in Cell Biology*, vol. 40, *A Practical Guide to the Study of Calcium in Living Cells*, ed. Nuccitelli, R., pp. 155–181. Academic Press, New York.

Katz, B. & Miledi, R. (1967). The timing of calcium action during neuromuscular transmission. *Journal of Physiology* **189**, 535–544.

Kuba, K. (1994). Ca^{2+}-induced Ca^{2+} release in neurones. *Japanese Journal of Physiology* **44**, 613–650.

KUBA, K., MORITA, K. & NOHMI, M. (1983). Origin of calcium ions involved in the generation of a slow afterhyperpolarization in bullfrog sympathetic neurons. *Pflügers Archiv* **399**, 194–202.

LEAL-CARDOSO, H., KOSCHORKE, G. M., TAYLOR, G. & WEINREICH, D. (1993). Electrophysiological properties and chemosensitivity of acutely isolated nodose ganglion neurons of the rabbit. *Journal of the Autonomic Nervous System* **45**, 29–39.

LIPSCOMBE, D., MADISON, D. V., POENIE, M., REUTER, H., TSIEN, R. W. & TSIEN, R. Y. (1988). Imaging of cytosolic Ca^{2+} transients arising from Ca^{2+} stores and Ca^{2+} channels in sympathetic neurons. *Neuron* **1**, 355–365.

LLANO, I., DIPOLO, R. & MARTY, A. (1994). Calcium-induced calcium release in cerebellar Purkinje cells. *Neuron* **12**, 663–673.

LÜSCHER, C. LIPP, P., LÜSCHER, H.-R. & NIGGLI, E. (1996). Control of action potential propagation by intracellular Ca^{2+} in cultured rat dorsal root ganglion cells. *Journal of Physiology* **490**, 319–324.

MAGEE, J. C. & SCHOFIELD, G. G. (1991). Room temperature culture extends the useful life of adult neurons for voltage-clamp experiments. *Journal of Neuroscience Methods* **38**, 201–208.

MARTY, A. (1989). The physiological role of calcium-dependent channels. *Trends in Neurosciences* **12**, 420–424.

MEI, N. (1983). Sensory structures in the viscera. In *Progress in Sensory Physiology*, vol. 4, ed. AUTRUM, H., OTTOSON, D., PERL, E. R., SCHMIDT, R. F., SHIMAZU, H. & WILLIS, W. D., pp. 1–42. Springer-Verlag, New York.

MILLER, R. J. (1987). Multiple calcium channels and neuronal function. *Science* **235**, 46–52.

NELSON, E. J., LI, C. C.-R., BANGALORE, R., BENSON, T., KASS, R. S. & HINKLE, P. M. (1994). Inhibition of L-type calcium-channel activity by thapsigargin and 2,5-t-butylhydroquinone, but not by cyclopiazonic acid. *Biochemical Journal* **302**, 147–154.

SAH, P. (1996). Ca^{2+}-activated K^+ currents in neurons: types, physiological roles and modulation. *Trends in Neurosciences* **11**, 150–154.

SCROGGS, R. S. & FOX, A. P. (1992). Multiple Ca^{2+} currents elicited by action potential waveforms in acutely isolated adult rat dorsal root ganglion neurons. *Journal of Neuroscience* **12**, 1789–1801.

SHMIGOL, A., VERKHRATSKY, A. & ISENBERG, G. (1995). Calcium-induced calcium release in rat sensory neurones. *Journal of Physiology* **489**, 627–636.

TATSUMI, H., HIRAI, K. & KATAYAMA, Y. (1988). Measurement of the intracellular calcium concentration in guinea-pig myenteric neurons by using fura-2. *Brain Research* **451**, 371–375.

THAYER, S. A., PERNEY, T. M. & MILLER, R. J. (1988). Regulation of calcium homeostasis in sensory neurons by bradykinin. *Journal of Neuroscience* **8**, 4089–4097.

WEINREICH, D. & WONDERLIN, W. F. (1987). Inhibition of calcium-dependent spike afterhyperpolarization increases excitability of rabbit visceral sensory neurones. *Journal of Physiology* **394**, 415–427.

WIER, W. G. (1990). Cytoplasmic $[Ca^{2+}]$ in mammalian ventricle: dynamic control by cellular processes. *Annual Review of Physiology* **52**, 467–485.

YOSHIZAKI, K., HOSHINO, T., SATO, M., KOYANO, H., NOHMI, M., HUA, S.-H. & KUBA, K. (1995). Ca^{2+}-induced Ca^{2+} release and its activation in response to a single action potential in rabbit otic ganglion cells. *Journal of Physiology* **486**, 177–187.

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