Ca^{2+} influx in resting rat sensory neurones that regulates and is regulated by ryanodine-sensitive Ca^{2+} stores

Yuriy M. Usachev and Stanley A. Thayer

Department of Pharmacology, University of Minnesota Medical School, 3-249 Millard Hall, 435 Delaware Street SE, Minneapolis, MN 55455, USA

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1. Store-operated, voltage-independent Ca^{2+} channels are activated by depletion of intracellular Ca^{2+} stores and mediate Ca^{2+} influx into non-excitable cells at resting membrane potential. We used microfluorimetry, patch-clamp and Mn^{2+}-quench techniques to explore the possibility that a similar mechanism exists in rat dorsal root ganglion (DRG) neurones in primary culture.

2. Following caffeine-induced depletion, ryanodine-sensitive Ca^{2+} stores refilled with Ca^{2+} at resting membrane potential. The refilling process required extracellular Ca^{2+}, was blocked by 2 mM Ni^{2+}, and was facilitated by membrane hyperpolarization from −55 to −80 mV, indicating a key role for Ca^{2+} influx. This influx of Ca^{2+} was not affected by the voltage-operated Ca^{2+} channel (VOCC) antagonists nicardipine (10 μM), nimodipine (10 μM) or ω-grammotoxin SIA (1 μM).

3. When ryanodine-sensitive Ca^{2+} stores were depleted in Ca^{2+}-free media, a return to 2 mM external Ca^{2+} resulted in a pronounced $[Ca^{2+}]_i$ overshoot, indicating an increased permeability to Ca^{2+}. Depletion of Ca^{2+} stores also produced a 2-fold increase in the rate of Mn^{2+} influx. The $[Ca^{2+}]_i$ overshoot and Mn^{2+} entry were both inhibited by Ni^{2+}, but not by VOCC antagonists.

4. Caffeine induced periodic Ca^{2+} release from, and reuptake into, ryanodine-sensitive stores. The $[Ca^{2+}]_i$ oscillations were arrested by removal of extracellular Ca^{2+} or by addition of Ni^{2+}, but they were not affected by VOCC antagonists. Hyperpolarization increased the frequency of this rhythmic activity.

5. These data suggest the presence of a Ca^{2+} entry pathway in mammalian sensory neurones that is distinct from VOCCs and is regulated by ryanodine-sensitive Ca^{2+} stores. This pathway participates in refilling intracellular Ca^{2+} stores and maintaining $[Ca^{2+}]_i$ oscillations and thus controls the balance between intra- and extracellular Ca^{2+} reservoirs in resting DRG neurones.

In non-excitable cells, capacitative Ca^{2+} influx is the principal route of Ca^{2+} entry (Berridge, 1995; Clementi & Meldolesi, 1996; Parekh & Penner, 1997). Store-operated Ca^{2+} channels (SOCCs) that are activated by depletion of intracellular Ca^{2+} stores mediate this influx. The single channel conductance of SOCCs is estimated to be below 1 pS (Hoth & Penner, 1993). SOCCs are selectively permeable to Ca^{2+} and blocked by a number of inorganic cations (Hoth & Penner, 1993), but no organic antagonist has proven to be selective for these channels (Lo & Thayer, 1995; Clementi & Meldolesi, 1996). SOCCs are voltage independent; thus, SOCC-mediated Ca^{2+} influx persists at resting membrane potential. Hyperpolarization increases Ca^{2+} influx through these channels due to the increased driving force for Ca^{2+} (Parekh & Penner, 1997). In non-excitable cells that lack voltage-gated Ca^{2+} channels, SOCCs are the primary pathway for the Ca^{2+} influx that is needed to replenish intracellular stores, to maintain $[Ca^{2+}]_i$ oscillations and to establish long-lasting increases in $[Ca^{2+}]_i$ following agonist-induced Ca^{2+} release (Berridge, 1995; Clementi & Meldolesi, 1996).

Voltage-operated Ca^{2+} channels (VOCCs) are the primary pathway for Ca^{2+} entry during excitation in neurones (Lipscombe *et al.* 1988; Thayer & Miller, 1990). These channels have been studied extensively in a variety of neuronal tissues (Bertolino & Llinás, 1992; Tsien *et al.* 1995). However, few studies have addressed the question of whether alternative routes of Ca^{2+} influx might contribute to $[Ca^{2+}]_i$ homeostasis in neurones at rest. The evidence for Ca^{2+} influx pathways active in quiescent neurones emerges from the observation that the $[Ca^{2+}]_i$ baseline is sensitive to the extracellular Ca^{2+} concentration (Lipscombe *et al.* 1988; Nohmi *et al.* 1992; Gomez *et al.* 1995). Furthermore, ryanodine-sensitive stores in sympathetic (Friel & Tsien, 1992), sensory (Usachev *et al.* 1993) and hippocampal neurones (Garaschuk *et al.* 1997) replenish spontaneously at resting membrane potential by a process that requires Ca^{2+}

influx. In neuroblastoma cells, Ca^{2+} influx was activated by agonist- or thapsigargin-induced depletion of Ca^{2+} stores (Takemura *et al.* 1991; Mathes & Thompson, 1995). Store depletion-activated Ca^{2+} influx has also been reported for other excitable cells such as phaeochromocytoma (PC12) cells (Clementi *et al.* 1992; Bennett *et al.* 1998) and pancreatic β-cells (Miura *et al.* 1997). Thus, store-operated Ca^{2+} influx may be a common feature of excitable cells.

In this report, we show that a mechanism similar to capacitative Ca^{2+} entry maintains Ca^{2+} influx in mammalian neurones at resting membrane potential. We used indo-1-based microfluorimetry in combination with whole-cell patch-clamp and Mn^{2+}-quench techniques to study Ca^{2+} entry into cultured rat dorsal root ganglion (DRG) neurones before and after depletion of intracellular Ca^{2+} stores. We found that Ca^{2+} influx persisted in resting neurones, was not prevented by selective antagonists of voltage-gated Ca^{2+} channels and increased with hyperpolarization. Depletion of Ca^{2+} stores increased Ca^{2+} influx. Ca^{2+} influx was needed to replenish ryanodine-sensitive stores and maintain caffeine-induced $[Ca^{2+}]_i$ oscillations. This pathway may regulate cellular Ca^{2+} levels between periods of electrical activity in neurones.

Preliminary reports of this work have been published previously (Usachev & Thayer, 1997; Thayer *et al.* 1998).

METHODS

Cell culture

Rat DRG neurones were grown in primary culture as described previously (Thayer & Miller, 1990). In brief, 1- to 3-day-old Sprague–Dawley rats were killed by decapitation with sharp scissors, according to a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). DRG were dissected from the thoracic and lumbar segments of the spinal cord and incubated at 37 °C in collagenase–dispase (0·8 and 6·4 U ml^{-1}, respectively) for 45 min. Ganglia were dissociated by trituration through a flame-constricted pipette and then plated onto laminin-coated (50 $\mu g\ ml^{-1}$) glass coverslips (25 mm diameter). Cells were grown in Ham's F12 medium supplemented with 5% heat-inactivated horse serum and 5% fetal bovine serum, 50 ng ml^{-1} nerve growth factor (NGF), 4·4 mM glucose, 2 mM L-glutamine, modified Eagle's medium vitamins, and penicillin–streptomycin (100 U ml^{-1} and 100 $\mu g\ ml^{-1}$, respectively). All cell culture reagents were purchased from Sigma. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were used within 2–6 days after plating.

Microfluorimetric measurements

$[Ca^{2+}]_i$ was recorded from cultured DRG neurones by using indo-1-based microfluorimetry (Grynkiewicz *et al.* 1985). The instrumentation has been described in detail previously (Werth *et al.* 1996). Cells were placed in a flow-through chamber (Thayer & Miller, 1990) (10 s solution exchange) that was mounted on the stage of an inverted epifluorescence microscope equipped with a ×70 objective (Leitz, NA = 1·15). Indo-1 was loaded into the cells by incubation in medium containing 5 μM indo-1 AM and 0·02% (w/w) Pluronic F-127 for 30 min at room temperature or via the patch pipette when the $[Ca^{2+}]_i$ measurements were combined with patch-clamp recordings. The dye was excited at 350 nm (10 nm bandpass) and emission was detected at 405 (20) and 490 (20) nm. Fluorescence was monitored by a pair of photomultiplier tubes (Thorn EMI, Fairfield, NJ, USA) operating in photon-counting mode. The 5 V output signals were then integrated by 8-pole Bessel filters and digitized at 1 or 10 Hz with an analog-to-digital converter (Indec Systems, Sunnyvale, CA, USA). Data were stored and analysed on an IBM-compatible computer.

$[Ca^{2+}]_i$ and Mn^{2+} influx were recorded simultaneously using fura-2-based microfluorimetry. The procedure for dye loading and the microscope configuration were similar to that used for indo-1. Fura-2 fluorescence was alternately excited at 360 (10) nm and 380 (10) nm by using a computer-controlled wheel (Sutter Instruments, Inc., Novato, CA, USA). Fluorescence images (510 nm, 40 nm bandpass) were projected onto a cooled charge-coupled device camera (Photometrics, Inc., Tucson, AZ, USA; 384 pixels × 576 pixels binned to 192 pixels × 288 pixels) controlled by an IBM-compatible computer. Image pairs were collected every 6 s and spatially averaged for further analysis.

Changes in the fluorescence of indo-1 (fura-2) were converted to $[Ca^{2+}]_i$ using the formula:

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

where R is the 405 nm/490 nm (360 nm/380 nm) fluorescence intensity ratio (Grynkiewicz *et al.* 1985). The dissociation constants used were 250 and 224 nM for indo-1 and fura-2, respectively. β is the ratio of fluorescence emitted at 490 nm for indo-1 or excited at 360 nm for fura-2 measured in the absence and presence of Ca^{2+}. R_{min}, R_{max} and β were determined in intact cells by applying 10 μM ionomycin in Ca^{2+}-free buffer (1 mM EGTA) and saturating Ca^{2+} (5 mM Ca^{2+}). Values for R_{min}, R_{max} and β, respectively, were 0·25, 2·3 and 3·5 for indo-1 and 1·0, 3·5 and 4·2 for fura-2. Mn^{2+} influx was measured as the change in fura-2 fluorescence excited near the isobestic point (360 nm). Background light levels were determined in an area that did not contain a cell.

Data are presented as means ± S.E.M.; n, number of cells tested. $P < 0{\cdot}05$ was considered significant.

Electrophysiology

Whole-cell patch-clamp recordings (Hamill *et al.* 1981) were obtained using a patch-clamp amplifier (PC501; Warner Instrument Corporation, Hamden, CT, USA) and an analog-to-digital converter (Indec Systems). The whole-cell currents were filtered at 1 kHz and sampled every 200 μs, or 100 ms when recorded in combination with measurements of $[Ca^{2+}]_i$. Patch pipettes were pulled from borosilicate glass (Narishige; 2–4 MΩ) on a Sutter Instruments P-87 micropipette puller and filled with the following solution (mM): potassium gluconate, 125; KCl, 10; Mg-ATP, 3; $MgCl_2$, 1; Hepes, 10; indo-1, 0·1; pH 7·25 with KOH, 290 mosmol kg^{-1} with sucrose. The extracellular recording solution contained (mM): NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; glucose, 10; pH 7·35 with NaOH, 310 mosmol kg^{-1} with sucrose. Ca^{2+}-free solution was obtained by substituting 0·1 mM EGTA for Ca^{2+}, unless otherwise indicated. To isolate Ca^{2+} currents from other currents, Cs^+ was substituted for K^+ in the pipette solution and extracellular Na^+ and K^+ were replaced with TEA^+; 10 mM BAPTA replaced indo-1 in the pipette solution when patch-clamp recordings were not accompanied by $[Ca^{2+}]_i$ measurements.

For the 'perforated' modification (Horn & Marty, 1988) of the patch-clamp technique the following solution was used in the patch pipettes (mM): KCl, 55; K_2SO_4, 70; $MgCl_2$, 7; Hepes, 10; pH 7·25 with KOH. Amphotericin B was added to filtered pipette solution from a DMSO stock (2 mg amphotericin B in 40 μl DMSO) to a final concentration of 240 $\mu g\ ml^{-1}$. The solution was kept in the dark and

used within 2 h. The access resistance became < 20 MΩ within 5–10 min after seal formation and was further compensated by 30–40%.

To evoke action potentials in intact neurones, extracellular field stimulation was employed (Werth *et al.* 1996). Field potentials were generated by passing current between two platinum electrodes via a Grass S44 stimulator and a stimulus isolation unit (Quincy, MA, USA). Trains of 1 ms pulses were delivered at a rate 10 Hz. The stimulus voltage sufficient to elicit a detectable increase in $[Ca^{2+}]_i$ from a cell was determined before beginning an experiment, and subsequent stimuli were 20 V over this threshold.

Reagents

Indo-1, indo-1 AM, fura-2 AM and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR, USA). ω-Grammotoxin SIA (GsTX) was a gift from Dr Richard A. Keith at Zeneca Pharmaceuticals. All other reagents were purchased from Sigma.

RESULTS

Ca^{2+} influx is required to refill ryanodine-sensitive Ca^{2+} stores

We have shown previously that ryanodine-sensitive Ca^{2+} stores refill spontaneously in resting DRG neurones (Usachev *et al.* 1993). However, a significant portion of released Ca^{2+} is extruded immediately from the cell by active transport (Benham *et al.* 1992; Usachev *et al.* 1993; Werth *et al.* 1996) reducing the level of intracellular Ca^{2+} in the cytosol available for subsequent replenishment of the stores. Therefore, an external source of Ca^{2+} is probably involved in the refilling process in resting neurones. We tested this hypothesis by studying the role of Ca^{2+} entry in refilling ryanodine-sensitive stores.

In this series of experiments, $[Ca^{2+}]_i$ was monitored in intact DRG neurones loaded with indo-1 AM. Caffeine (5 mM), a modulator of ryanodine receptors, was employed in a paired-pulse protocol in which the first (control) application of caffeine was used to deplete the stores, then after a delay, a second (test) caffeine stimulation was applied to probe the amount of Ca^{2+} that had reaccumulated in the stores (Fig. 1*A*). During depolarization, Ca^{2+} influx through VOCCs supplies Ca^{2+} to reload intracellular Ca^{2+} stores (Usachev *et al.* 1993; Garaschuk *et al.* 1997). In these experiments, a complete replenishment of the Ca^{2+} stores was provided before each pair of caffeine applications by a series of action potentials (10 Hz for 5 s) using extracellular field stimulation. Caffeine was added to Ca^{2+}-free media to prevent Ca^{2+} influx during the release activation. The amplitude of the test $[Ca^{2+}]_i$ response was normalized to the amplitude of the control response and used as an index of the efficiency of the refilling process. This paired-pulse protocol renders the peak $[Ca^{2+}]_i$ dependent on the refilling state of the stores because basal $[Ca^{2+}]_i$ was constant (± 20 nM) for a given pair of stimuli and $[Ca^{2+}]_i$ buffering processes were much slower (the time from peak to basal $[Ca^{2+}]_i$ was 165 ± 14 s) than the upstroke of the $[Ca^{2+}]_i$ transient (the time from basal to peak $[Ca^{2+}]_i$ was 24 ± 3 s; $n = 16$), providing a clear temporal separation of release from recovery. The Ca^{2+} stores refilled in a time-dependent manner. For example, the amplitude of the test response after 1 min of refilling was only $24 \pm 9\%$ ($n = 6$) of control, whereas after 20 min the test response recovered to $86 \pm 6\%$ of control ($n = 8$). The refilling was completely blocked ($n = 4$, data not shown) by 1 μM cyclopiazonic acid (CPA), a selective antagonist of sarco–endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) (Thomas & Hanley, 1994). This is consistent with the role of SERCAs in actively transporting Ca^{2+} into intracellular stores (Pozzan *et al.* 1994).

Next, we tested whether replenishment of ryanodine-sensitive stores depended on extracellular Ca^{2+}. Removal of extracellular Ca^{2+} during the interval between caffeine applications blocked refilling, as indicated by the failure of the second application of caffeine to evoke a $[Ca^{2+}]_i$ response ($n = 4$; Fig. 1*B*). The response did not recover if the cell remained in Ca^{2+}-free buffer (data not shown), suggesting that Ca^{2+} influx is needed to refill the stores. Furthermore, if the cell was treated with 2 mM Ni^{2+} during the refilling interval, as shown in Fig. 1*C*, the amplitude of the test $[Ca^{2+}]_i$ response decreased from 69 ± 5 to $28 \pm 5\%$ of control ($n = 18$; $P < 0{\cdot}001$, Student's paired t test). The inhibitory effect of Ni^{2+} was fully reversible.

Ca^{2+} influx at resting membrane potential is not mediated by voltage-gated Ca^{2+} channels

Because the refilling of Ca^{2+} stores required Ca^{2+} influx, we tried to determine which channels mediated Ca^{2+} influx in unstimulated neurones. In non-excitable cells this influx is conducted by SOCCs (Berridge, 1995; Parekh & Penner, 1997). In neurones, VOCCs are the principal Ca^{2+} entry pathway during electrical activity (Lipscombe *et al.* 1988; Thayer & Miller, 1990). However, the contribution of VOCCs to Ca^{2+} entry at resting membrane potential is not clear. We investigated whether Ca^{2+} influx in resting DRG neurones was mediated by VOCCs.

DRG neurones express T-, L-, N- and P/Q-type Ca^{2+} channels (Fox *et al.* 1987; Rusin & Moises, 1995). We studied the effects of VOCC antagonists on the refilling of Ca^{2+} stores by using a paired-pulse protocol in which the caffeine stimuli were separated by 5 min intervals (Figs 2 and 3). Nimodipine and GsTX were used in combination to block L-type channels and N- and P/Q-type channels, respectively (McCarthy & TanPiengco, 1992; Piser *et al.* 1995). In these experiments, the normalized amplitude of the test $[Ca^{2+}]_i$ response was $66 \pm 5\%$ ($n = 8$) in untreated cells and was dependent on Ca^{2+} influx, as indicated by sensitivity to Ni^{2+} (2 mM). However, the combination of 10 μM nimodipine and 1 μM GsTX did not affect refilling of the stores (Fig. 2*A*) and the normalized amplitude of the test response after this treatment was $71 \pm 6\%$ ($n = 8$). The same combination of drugs completely blocked the $[Ca^{2+}]_i$ response elicited by depolarization with 40 mM K^+ ($n = 5$; Fig. 2*B*) and inhibited by $94 \pm 3\%$ ($n = 6$) high-threshold voltage-gated Ca^{2+} currents elicited by step depolarization from −60 to +10 mV (Fig. 2*C*).

We next examined the contribution of T-type Ca^{2+} channels to the refilling of ryanodine-sensitive Ca^{2+} stores. We found that amiloride (500 μM), which is often used to block low-threshold voltage-gated Ca^{2+} channels, strongly interfered with indo-1 fluorescence ($n = 4$; data not shown) and therefore could not be used for $[Ca^{2+}]_i$ measurements. Certain dihydropyridines have been reported to block T-type channels at high concentrations (Akaike *et al.* 1989). In DRG neurones, nicardipine was shown to be effective (Richard *et al.* 1991). In our system, 10 μM nicardipine inhibited by $97 \pm 3\%$ ($n = 5$) low-threshold voltage-gated currents elicited by step depolarization from −90 to −30 mV (Fig. 3*B*). The effect of nicardipine reversed slowly with time. For comparison, 500 μM amiloride and 100 μM Ni^{2+} reduced low-threshold Ca^{2+} currents by $64 \pm 3\%$ ($n = 4$) and $88 \pm 3\%$ ($n = 5$), respectively (data not shown). However, 10 μM nicardipine did not slow the refilling process (Fig. 3*A*). In these experiments, the normalized amplitude of the test $[Ca^{2+}]_i$ response was $71 \pm 13\%$ ($n = 4$) in untreated cells and $77 \pm 11\%$ ($n = 4$) after treatment with 10 μM nicardipine.

Voltage dependence can reveal important information about the nature of ionic fluxes. Hyperpolarization would be predicted to decrease Ca^{2+} influx mediated by VOCCs due to decreased channel activation (Fox *et al.* 1987; Magee *et al.* 1996). In contrast, SOCC-mediated Ca^{2+} influx would be greater at more negative potentials because the driving force for Ca^{2+} would increase without a decrease in channel opening (Parekh & Penner, 1997). The effects of changes in membrane potential during the refilling process are described in Fig. 4. The paired-pulse protocol was applied to neurones clamped at −55 or −80 mV using the whole-cell configuration of the patch-clamp technique. Because the stability of the electrical recording was impaired in Ca^{2+}-free media (0·1 mM EGTA), extracellular Ca^{2+} was not removed during caffeine applications in these experiments. When cells were clamped at −55 mV during the interval between two caffeine applications, the normalized amplitude

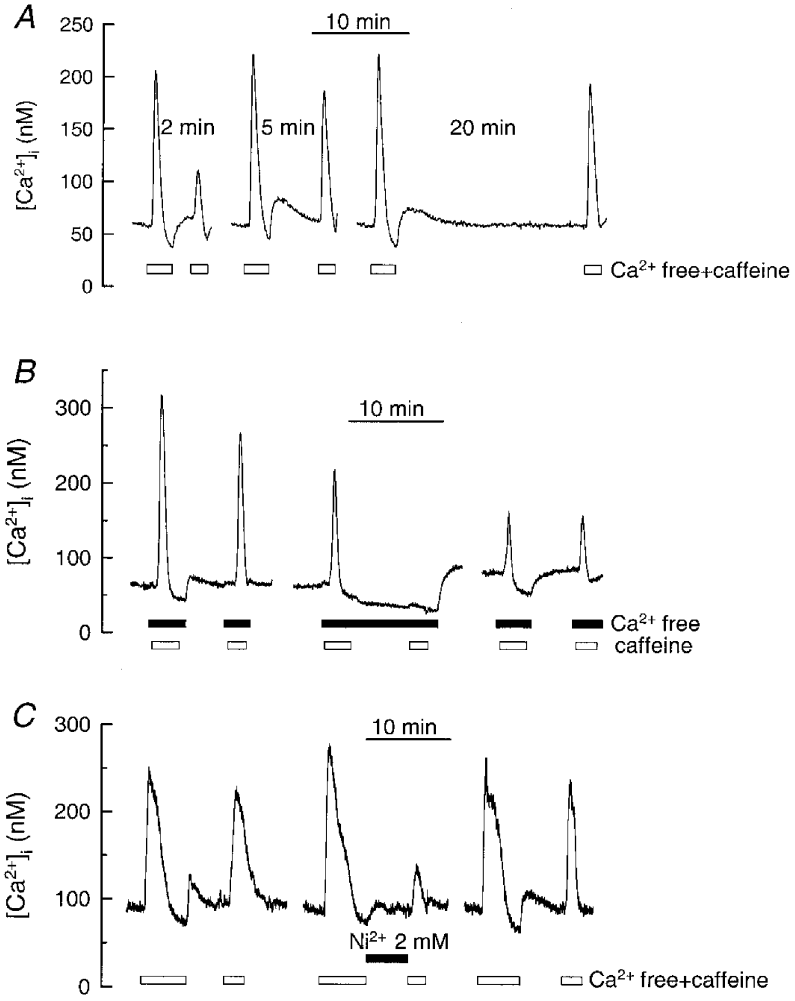

Figure 1. Ca^{2+} influx is required to refill ryanodine-sensitive Ca^{2+} stores

A, Ca^{2+} release was activated by 5 mM caffeine in Ca^{2+}-free extracellular solution, as indicated by the horizontal bars. The paired $[Ca^{2+}]_i$ transients are presented in order of increasing interstimulus interval as shown above the $[Ca^{2+}]_i$ traces, although they were intermixed in the actual experiment. In *B* and *C*, the effect of extracellular Ca^{2+} (*B*) and Ni^{2+} (*C*) on the refilling process was studied using a paired-pulse protocol. The interval between two subsequent caffeine applications was 5 min. The horizontal bars indicate the duration of the treatments.

of the test $[Ca^{2+}]_i$ response was $65 \pm 6\%$ of the control ($n = 8$). Hyperpolarization to −80 mV significantly facilitated filling of the Ca^{2+} stores and the amplitude of the test response increased to $90 \pm 7\%$ ($n = 8$; Fig. 4*B*). This facilitation was probably a result of enhanced Ca^{2+} influx. Indeed, 40 s hyperpolarization from −55 to −80 mV following depletion of the stores with 10 mM caffeine produced a small elevation in $[Ca^{2+}]_i$ that could be detected in the presence of 5 mM Ca^{2+} in the extracellular solution (Fig. 4*C*). Ni^{2+} (2 mM) inhibited this elevation, reducing the amplitude of the response from 44 ± 11 to 15 ± 2 nM ($n = 5$). Similar results were obtained using the amphotericin B-based patch-clamp technique ($n = 4$; data not shown). These data demonstrate that in DRG neurones significant Ca^{2+} influx occurs at resting membrane potential that is independent of VOCCs. We next explored the possibility that SOCCs might mediate this Ca^{2+} influx. If SOCCs are present in these cells then Ca^{2+} influx might be increased by depletion of intracellular Ca^{2+} stores.

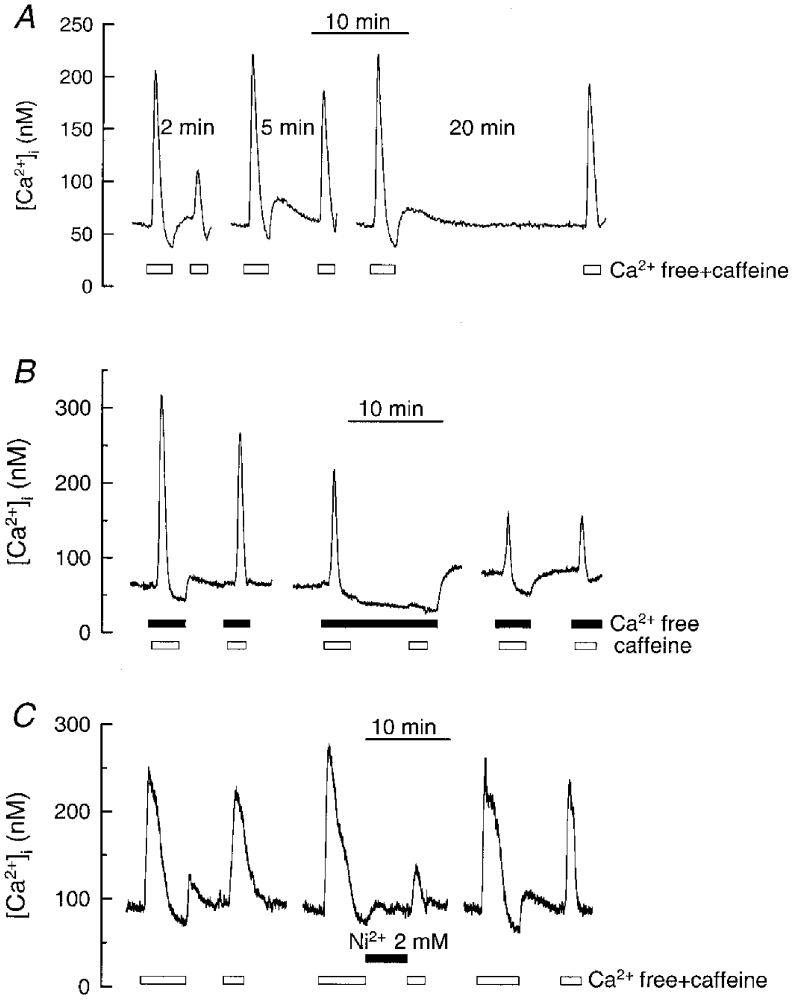

Figure 2. Refilling of ryanodine-sensitive Ca^{2+} stores is not dependent on high-threshold voltage-gated Ca^{2+} channels

A, $[Ca^{2+}]_i$ transients were elicited by 5 mM caffeine in Ca^{2+}-free media. The cells were treated with a mixture of 10 μM nimodipine (Nim) and 1 μM ω-grammotoxin SIA (GsTX) during the 5 min period between two subsequent applications of caffeine. The duration of the drug application is indicated by the horizontal bars below the $[Ca^{2+}]_i$ traces. *B*, $[Ca^{2+}]_i$ transients were elicited by 40 mM K^+. The combination of 10 μM nimodipine and 1 μM GsTX completely blocked depolarization-induced increases in $[Ca^{2+}]_i$. The break in the $[Ca^{2+}]_i$ trace corresponds to 20 min. *C*, whole-cell Ca^{2+} currents were evoked by depolarization from −60 to +10 mV for 100 ms. Voltage pulses were applied every 30 s. The amplitude of the Ca^{2+} current is plotted *versus* time. The traces (right) show currents for the same experiments obtained before treatment (control) and after block with a combination of 10 μM nimodipine and 1 μM GsTX.

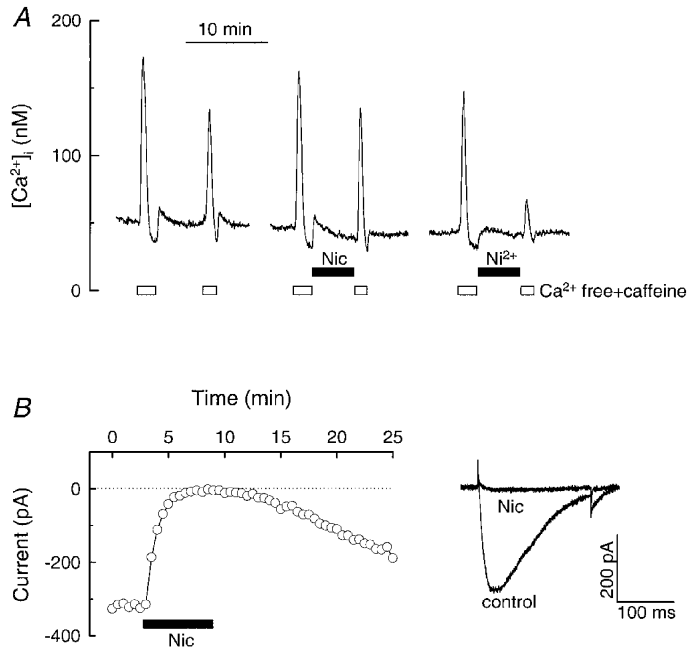

Figure 3. Low-threshold voltage-gated Ca^{2+} channels do not mediate refilling of ryanodine-sensitive Ca^{2+} stores

A, $[Ca^{2+}]_i$ transients were elicited by 5 mM caffeine in Ca^{2+}-free media. Nicardipine (10 μM; Nic) was applied during the 5 min period between the two caffeine applications as indicated by the filled horizontal bar. *B*, whole-cell Ca^{2+} currents were evoked by depolarization from −90 to −30 mV for 200 ms. Voltage pulses were applied every 30 s. The amplitude of the Ca^{2+} current is plotted *versus* time. The current traces (right) represent Ca^{2+} currents before (control) and during treatment of the cell with 10 μM nicardipine.

Depletion of intracellular Ca^{2+} stores facilitates Ca^{2+} influx in resting neurones

In non-excitable cells, store-operated Ca^{2+} influx is commonly exhibited as a sustained plateau phase following agonist-evoked Ca^{2+} release from intracellular stores. This plateau phase depends on extracellular Ca^{2+} and is thought to be mediated by store-operated Ca^{2+} channels (Jacob, 1990; Parekh & Penner, 1997). We found that in DRG neurones a rapid caffeine-induced $[Ca^{2+}]_i$ rise was followed by a sustained $[Ca^{2+}]_i$ elevation above the baseline (Fig. 5*A*). The amplitude of the initial $[Ca^{2+}]_i$ increase did not depend on extracellular Ca^{2+} (172 ± 16 nM, $n = 10$, with 2 mM Ca^{2+}

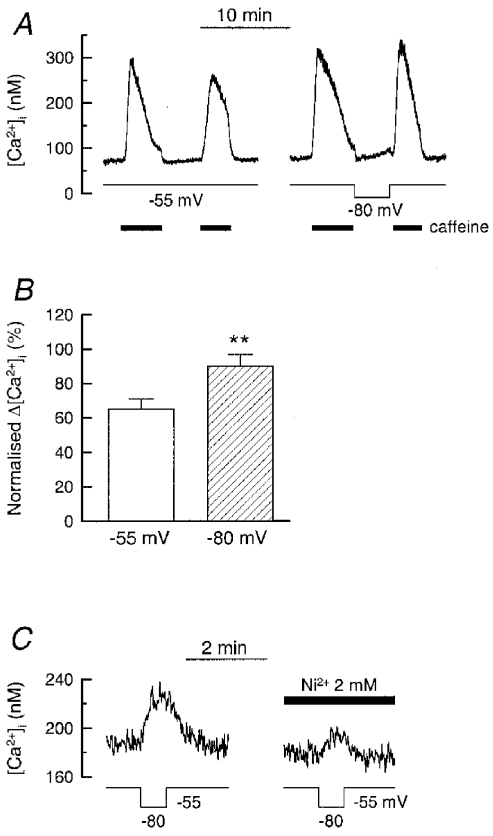

Figure 4. Hyperpolarization increases Ca^{2+} influx and facilitates the refilling process

DRG neurones were loaded with 100 μM indo-1 using the patch pipette. *A*, $[Ca^{2+}]_i$ was measured in neurones clamped to −55 or −80 mV following stimulation with 5 mM caffeine, as indicated below the $[Ca^{2+}]_i$ traces. For this paired-pulse protocol, the interstimulus time was 5 min. *B*, histogram displaying the amplitude of the second $[Ca^{2+}]_i$ response normalized to the first for a paired-pulse protocol such as that described in *A* ($n = 8$). The membrane potential in the interval between the two subsequent caffeine applications is indicated below the bars. ** $P < 0{\cdot}01$, Student's paired t test. *C*, hyperpolarization induced an elevation in $[Ca^{2+}]_i$ that was inhibited by 2 mM Ni^{2+}. In both instances, hyperpolarization was applied after the stores were depleted with 10 mM caffeine. The extracellular Ca^{2+} concentration was increased to 5 mM.

in the media, and 186 ± 16 nM, $n = 10$, in Ca^{2+}-free media) consistent with Ca^{2+} release from intracellular stores. In contrast, the plateau phase could be abolished by removal of extracellular Ca^{2+} ($n = 10$), suggesting that it was mediated by Ca^{2+} influx. In the absence of extracellular Ca^{2+}, caffeine-induced $[Ca^{2+}]_i$ transients recovered completely to the baseline within 3 min, whereas in control (2 mM Ca^{2+} in the media) $[Ca^{2+}]_i$ was elevated 47 ± 13 nM ($n = 10$) and 9 ± 3 nM ($n = 10$) above the baseline when measured 3 and 8 min, respectively, after the beginning of the response (Fig. 5*B*). Caffeine quenches indo-1 fluorescence, although this effect is wavelength independent and does not influence the ratiometric measurements (O'Neill *et al.* 1990). Thus, the observed plateau phase was unlikely to result from an indo-1–caffeine interaction. Furthermore, a similar sustained $[Ca^{2+}]_i$ elevation was observed with another Ca^{2+}-sensitive dye, fura-2 ($n = 6$, data not shown), the fluorescence of which increases in the presence of caffeine (Nohmi *et al.* 1992). The plateau phase displayed a graded inactivation. This may result from Ca^{2+}-dependent inhibition of Ca^{2+} influx (Zweifach & Lewis, 1995; Madge *et al.* 1997), inactivation of ryanodine receptor-mediated Ca^{2+} release with subsequent store refilling (Pozzan *et al.* 1994), or an upregulation of Ca^{2+} efflux processes (Miller, 1991). Depletion of Ca^{2+} stores with CPA also produced a sustained elevation in $[Ca^{2+}]_i$; it decreased at a slower rate (plateau amplitude at 8 min = 63 ± 15 nM; $n = 7$), suggesting that inactivation of Ca^{2+} release contributed to the decline in $[Ca^{2+}]_i$ during the plateau phase.

Store-operated Ca^{2+} influx in non-excitable cells can also be observed as a pronounced $[Ca^{2+}]_i$ increase elicited by switching from Ca^{2+}-free to Ca^{2+}-containing media following depletion of intracellular Ca^{2+} stores (Clementi *et al.* 1992; Berridge, 1995; Bennett *et al.* 1998). Depletion of the stores opens plasmalemmal channels that allow Ca^{2+} to rush into the cell upon its return to the media. We found

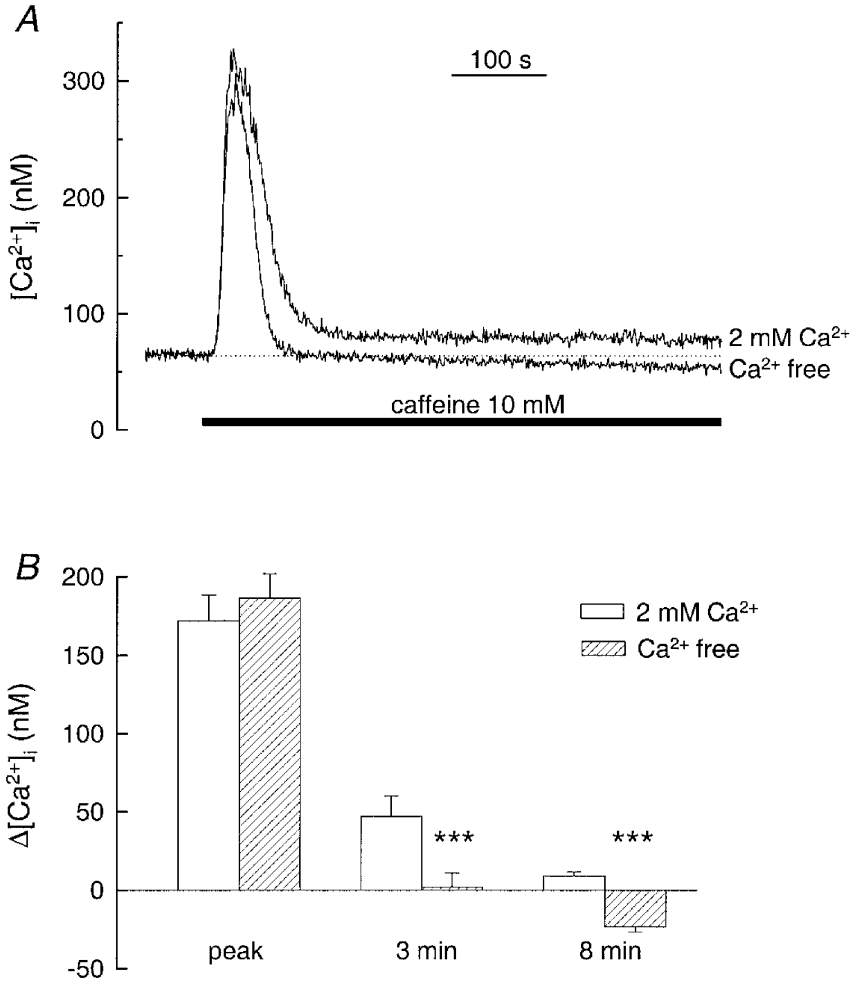

Figure 5. Caffeine-induced $[Ca^{2+}]_i$ response exhibits a Ca^{2+} influx-dependent plateau phase

A, $[Ca^{2+}]_i$ was measured in indo-1 AM-loaded DRG neurones during caffeine (10 mM) application. Representative $[Ca^{2+}]_i$ traces obtained in the presence of 2 mM extracellular Ca^{2+} (2 mM Ca^{2+}) or in Ca^{2+}-free media were superimposed. *B*, histogram displaying the difference between $[Ca^{2+}]_i$ and the baseline measured at the peak of the $[Ca^{2+}]_i$ response, and at 3 and 8 min after the beginning of the response ($n = 10$). The baseline was measured before drug application. Data obtained in the presence or absence of extracellular Ca^{2+} were compared using Student's paired t test (*** $P < 0{\cdot}001$). Negative values correspond to $[Ca^{2+}]_i$ levels below baseline.

that readdition of external Ca^{2+} to the bath after caffeine-induced discharge of the stores resulted in a $[Ca^{2+}]_i$ overshoot in 68% of DRG neurones tested ($n = 47$; Fig. 6), which is consistent with the presence of store-operated Ca^{2+} entry in neurones. During this transient elevation, $[Ca^{2+}]_i$ peaked at 37 ± 6 nM above resting $[Ca^{2+}]_i$ ($n = 28$) and the maximal rate of $[Ca^{2+}]_i$ rise was 4 ± 1 nM s^{-1} ($n = 28$). $[Ca^{2+}]_i$ then recovered to the basal level. The recovery could be fitted by a monoexponential function with a time constant of 123 ± 7 s ($n = 28$). Ni^{2+} (2 mM), but not the selective VOCC antagonists, inhibited the overshoot by 62 ± 6% ($n = 8$; Fig. 6*C*, see also Figs 1*C*, 2*A* and 3*A*). The overshoot was not observed if the switch from Ca^{2+}-free to Ca^{2+}-containing media (2 mM Ca^{2+}) was not preceded by depletion of the stores ($n = 6$; Fig. 6*A*).

In addition to its action on ryanodine receptors, caffeine is also known to inhibit phosphodiesterases and will thus increase the intracellular concentrations of cyclic AMP and cyclic GMP (Daly, 1993). These cyclic nucleotides may affect the Ca^{2+} permeability of the plasma membrane (Clementi & Meldolesi, 1996). To address the question of whether the action of caffeine resulted from an increase in cyclic nucleotides, we treated cells with 0·5 mM 3-isobutyl-1-methylxanthine (IBMX), which is an approximately 30-fold more potent inhibitor of phosphodiesterases than caffeine (Daly, 1993), but will not release Ca^{2+} from stores at this

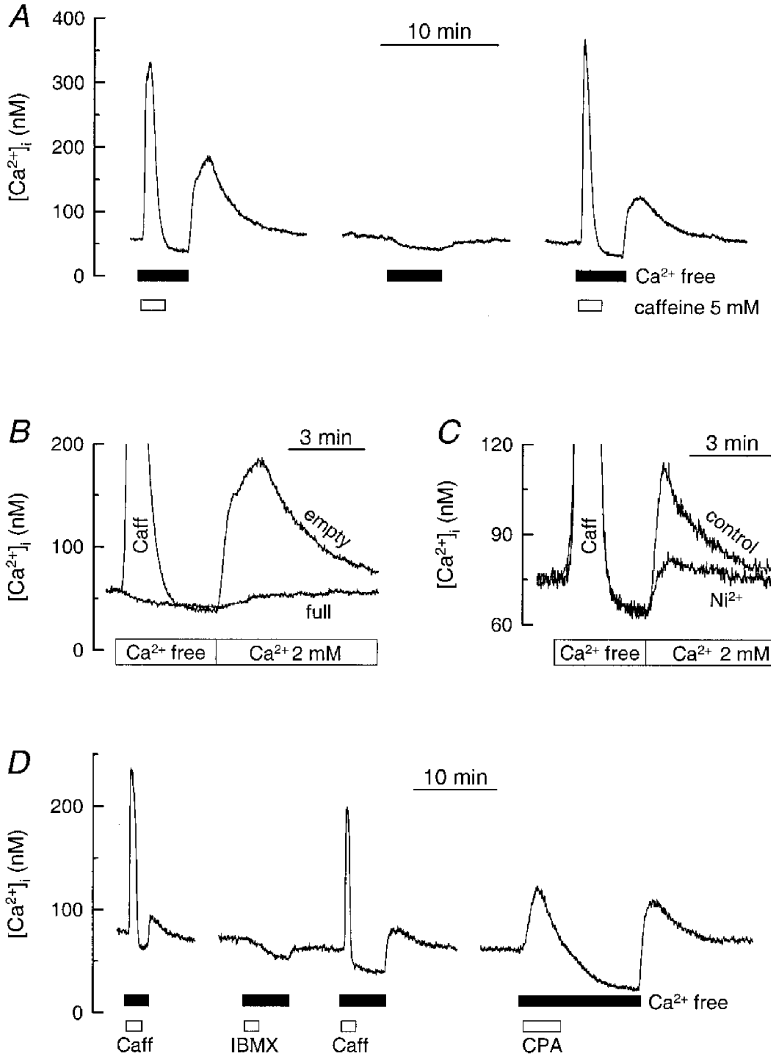

Figure 6. Depletion of intracellular Ca^{2+} stores increases Ca^{2+} influx

$[Ca^{2+}]_i$ was measured in intact neurones loaded with indo-1 AM. *A*, a $[Ca^{2+}]_i$ overshoot was observed after depletion of ryanodine-sensitive stores with caffeine. The breaks between the traces correspond to 15 min during which the cell was stimulated with 3 trains of action potentials (4 s at 10 Hz) elicited by extracellular field stimulation. *B*, for the recordings shown in *A*, changes in $[Ca^{2+}]_i$ evoked by addition of 2 mM Ca^{2+} following store depletion with caffeine (empty) or with 'full' stores are superimposed. The caffeine-elicited $[Ca^{2+}]_i$ transient is indicated (Caff). In *C*, the $[Ca^{2+}]_i$ overshoots elicited by store depletion in the absence (control) or presence (Ni^{2+}) of 2 mM Ni^{2+} are compared. Application of 5 mM caffeine is indicated on the trace (Caff). *D*, a $[Ca^{2+}]_i$ overshoot was elicited by depletion of the stores with 5 mM caffeine or 5 μM cyclopiazonic acid (CPA), but not by 0·5 mM 3-isobutyl-1-methylxanthine (IBMX). Horizontal bars below the trace indicate the duration of treatment.

concentration (Usachev & Verkhratsky, 1995). As shown in Fig. 6*D*, treatment with 0·5 mM IBMX did not produce a $[Ca^{2+}]_i$ overshoot ($n = 3$). Furthermore, the induction of the overshoot did not depend on the delay between removal of caffeine and addition of Ca^{2+} to the external media. Thus, the overshoot resulted from depletion of Ca^{2+} stores and subsequent activation of Ca^{2+} influx. An alternative means of depleting Ca^{2+} stores is to inhibit the SERCAs with agents such as CPA (Thomas & Hanley, 1994). $[Ca^{2+}]_i$ transients elicited by CPA (5 μM) developed more slowly and had a smaller amplitude than those induced by caffeine (Figs 6*D* and 9*A*). To ensure an effective discharge of the stores we applied CPA for 4–8 min (compare with 2–3 min for caffeine). After treatment with 5 μM CPA in Ca^{2+}-free media, addition of 2 mM Ca^{2+} elicited a pronounced overshoot in $[Ca^{2+}]_i$ in five of six neurones tested (Fig. 6*D*). The amplitude of the overshoot relative to the $[Ca^{2+}]_i$ baseline was 75 ± 23 nM ($n = 5$).

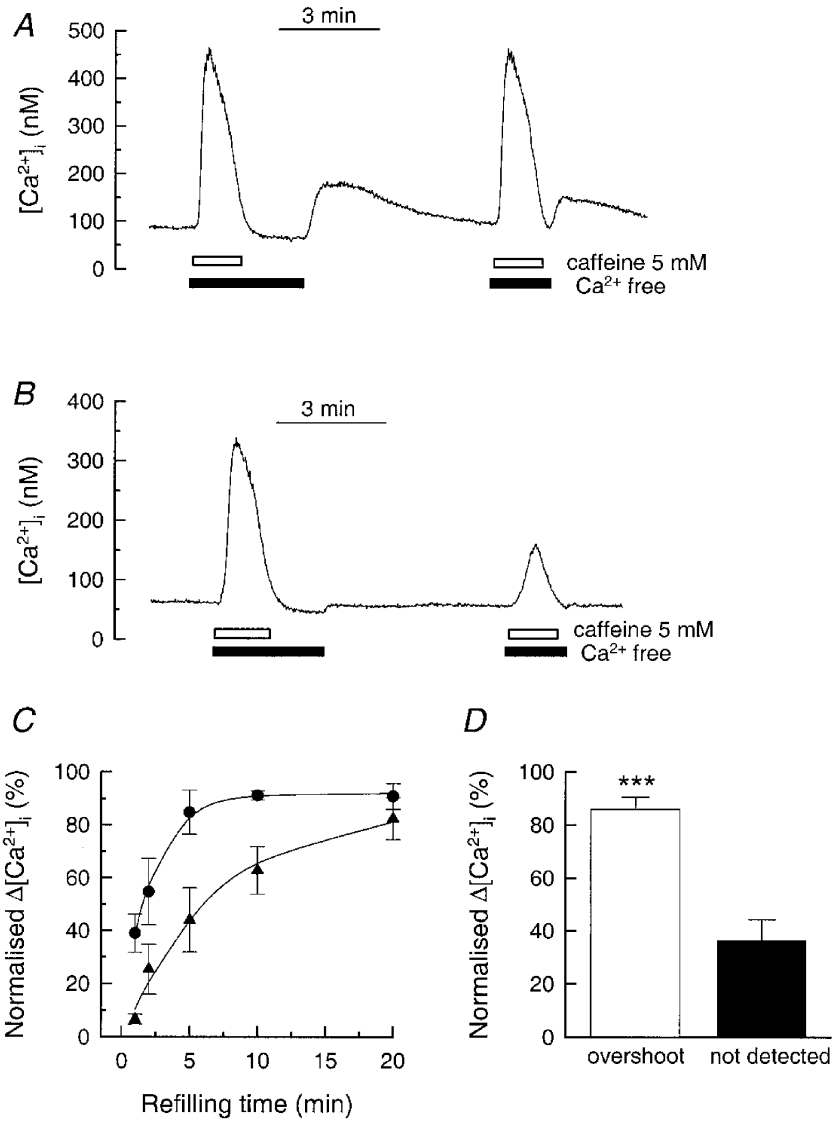

Figure 7. Ryanodine-sensitive stores refill faster in neurones that exhibit a $[Ca^{2+}]_i$ overshoot

A and *B*, $[Ca^{2+}]_i$ transients were elicited by 5 mM caffeine in DRG neurones with (*A*) or without (*B*) an overshoot, to test the rate of refilling of the stores using a paired-pulse protocol. The interstimulus time was 5 min. In *C*, the time dependence of the refilling process was studied using a paired-pulse protocol in neurones with (●; $n = 4$) or without (▲; $n = 5$) a $[Ca^{2+}]_i$ overshoot. An overshoot was defined as a net $[Ca^{2+}]_i$ increase greater than 10 nM that recovered to the basal level within 5–10 min. Ryanodine-sensitive Ca^{2+} stores were depleted by the first (control) application of 5 mM caffeine in Ca^{2+}-free media. The level of replenishment of the Ca^{2+} stores at a given time was evaluated by applying a second (test) stimulus with 5 mM caffeine in Ca^{2+}-free media. Each point represents the mean ± S.E.M. of the test response normalized to the control for various interstimulus time intervals. Data points were fitted with a single exponential function (smooth curves) using a non-linear, least-squares curve fitting algorithm (Origin software, Microcal). In *D*, the normalized amplitudes of the test response for neurones with ($n = 21$; overshoot) and without ($n = 12$; not detected) a $[Ca^{2+}]_i$ overshoot are compared for experiments such as those described in *A* and *B*. *** $P < 0{\cdot}001$; Student's unpaired *t* test.

These data suggest that the overshoot reflects facilitation of Ca^{2+} influx after depletion of Ca^{2+} stores that, in turn, should accelerate replenishment of the stores. We compared the rate of refilling of the Ca^{2+} stores in cells that displayed an overshoot (Fig. 7*A*) with that in cells in which the overshoot could not be detected (Fig. 7*B*). As summarized in Fig. 7*C* and *D*, the replenishment occurred significantly faster in cells for which Ca^{2+} influx was facilitated by store depletion. Using a paired-pulse protocol (Fig. 1*A*), we found that the refilling process could be described by a monoexponential function with a time constant of 137 s for cells with the overshoot and 414 s for those without it (Fig. 7*C*). Furthermore, the amplitudes of the caffeine-induced $[Ca^{2+}]_i$ response after 5 min of refilling recovered to $85 \pm 4\%$ ($n = 21$) and $36 \pm 8\%$ ($n = 12$) for the cells with and without overshoot, respectively (Fig. 7*D*).

Mn^{2+} permeates many Ca^{2+} pathways and, because it quenches the fluorescence of the Ca^{2+} indicator fura-2, it can be used as a probe for divalent cation entry (Jacob, 1990). We measured Mn^{2+} influx in intact neurones as a decrease in fura-2 fluorescence excited near the isobestic point for Ca^{2+} (360 nm). $[Ca^{2+}]_i$ was monitored simultaneously by

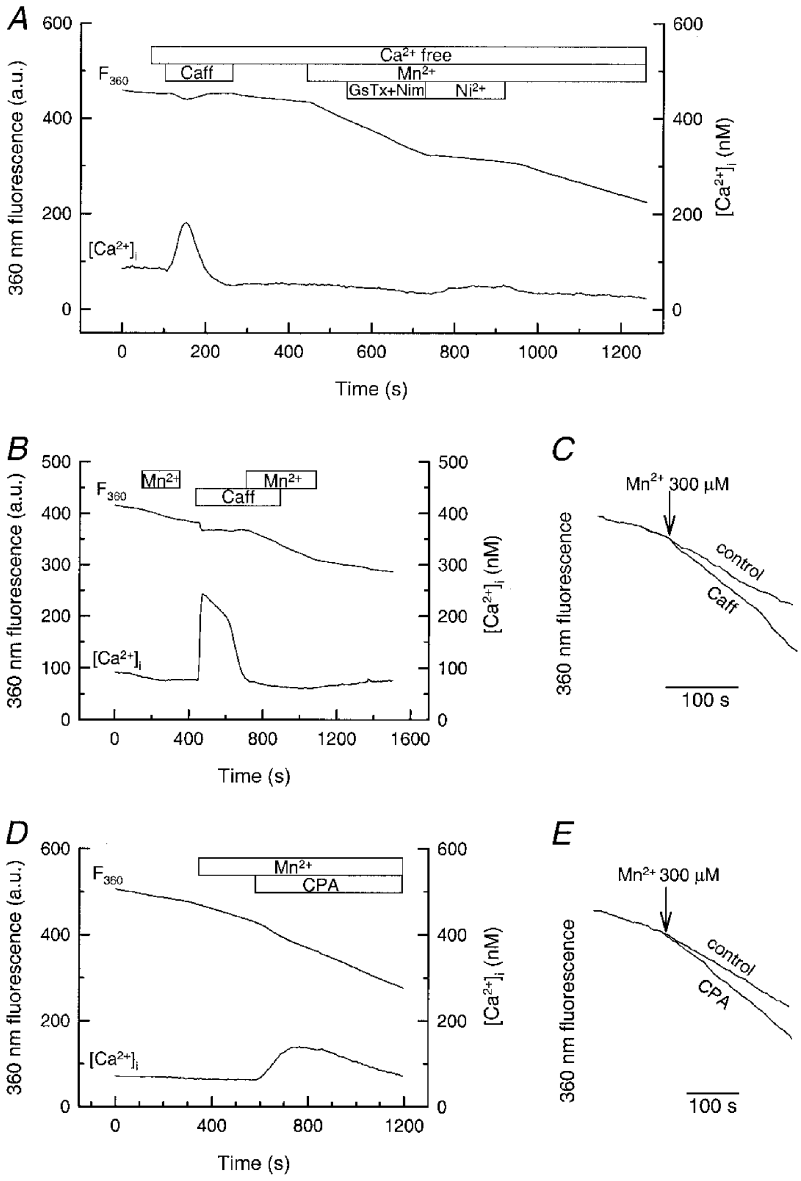

Figure 8. Depletion of Ca^{2+} stores increases the rate of Mn^{2+} influx in DRG neurones

$[Ca^{2+}]_i$ and Mn^{2+} (300 μM extracellular concentration) influx were measured simultaneously in fura-2 AM-loaded DRG neurones. Mn^{2+} influx was detected as a quench of 360 nm fluorescence (F_{360}) shown in arbitrary units (a.u.). *A*, to maximize Mn^{2+} influx, Ca^{2+} stores were depleted with 5 mM caffeine (Caff) in Ca^{2+}-free media. Mn^{2+} influx was slowed significantly by 2 mM Ni^{2+} but not by combined application of the VOCC antagonists GsTX (1 μM) and nimodipine (10 μM; Nim). Horizontal bars above the traces indicate the duration of the drug treatments. *B–E*, depletion of Ca^{2+} stores with 5 mM caffeine (*B* and *C*) or 10 μM CPA (*D* and *E*) increased the influx of Mn^{2+}. The corresponding traces before (control) and after (*C*, Caff; *E*, CPA) depletion of the stores were offset along the fluorescence intensity axis and compared in *C* and *E*.

taking the ratio of the fluorescence signals excited at 360 and 380 nm (F_{360}/F_{380}). Figure 8*A* shows an example of such a recording. In untreated cells, F_{360} decreased slowly with time ($0{\cdot}07 \pm 0{\cdot}01$ arbitrary units per second (a.u. s^{-1}); $n = 15$) presumably as a result of fura-2 bleaching. To maximize Ca^{2+} influx, Ca^{2+} stores were depleted by application of 5 mM caffeine in Ca^{2+}-free buffer. Addition of 300 μM Mn^{2+} increased significantly the rate of decay of F_{360} ($0{\cdot}39 \pm 0{\cdot}04$ a.u. s^{-1}; $n = 11$). A mixture of the VOCC antagonists GsTX (1 μM) and nimodipine (10 μM) did not change the Mn^{2+} influx, whereas it was inhibited by 2 mM Ni^{2+} (Fig. 8*A*); the F_{360} decay rate was slowed to $0{\cdot}12 \pm 0{\cdot}02$ a.u. s^{-1} ($n = 11$). Nicardipine (10 μM) was also without effect ($n = 4$; data not shown). These data are consistent with our previous observations (Figs 2*A*, 3*A* and 6*C*) and suggest that Mn^{2+} can be used as a probe for Ca^{2+} influx in neurones. Next we compared the rate of Mn^{2+} influx before and after depletion of Ca^{2+} stores by 5 mM caffeine (Fig. 8*B* and *C*) or 10 μM CPA (Fig. 8*D* and *E*). Both treatments significantly increased the F_{360} decay rate ($P < 0{\cdot}01$ and $< 0{\cdot}05$, respectively; Student's paired *t* test). After correcting for background decay of the F_{360} signal caused by fura-2 bleaching, we found that depletion of the stores with 5 mM caffeine or 10 μM CPA increased the rate of Mn^{2+} influx by $2{\cdot}3 \pm 0{\cdot}5$-fold ($n = 6$) and $2{\cdot}0 \pm 0{\cdot}4$-fold ($n = 5$), respectively.

The state of ryanodine-sensitive stores controls the size and the shape of $[Ca^{2+}]_i$ transients elicited by trains of action potentials

We have shown that store-regulated Ca^{2+} influx is the principal source for refilling ryanodine-sensitive Ca^{2+} stores at the resting membrane potential in rat DRG neurones. When Ca^{2+} influx was blocked, intracellular Ca^{2+} stores remained empty. Ca^{2+} stores can regulate $[Ca^{2+}]_i$ responses evoked by K^+ application in neurones (Friel & Tsien, 1992; Usachev *et al.* 1993). We compared Ca^{2+} signals in response to physiological stimuli for full and empty stores (Fig. 9*A*). Trains of action potentials were elicited every 60 or 120 s (indicated by the triangles) by extracellular field stimulation (10 Hz) to produce $[Ca^{2+}]_i$ transients in intact cells. For a given cell, the intensity of the stimulus remained constant during the recording. The amplitude ($\Delta[Ca^{2+}]_i$) and the time constant for the monoexponential function that described the recovery process (τ) for each $[Ca^{2+}]_i$ transient were analysed. Depletion of the stores by 5 mM caffeine significantly reduced the time constant from 12 ± 3 to

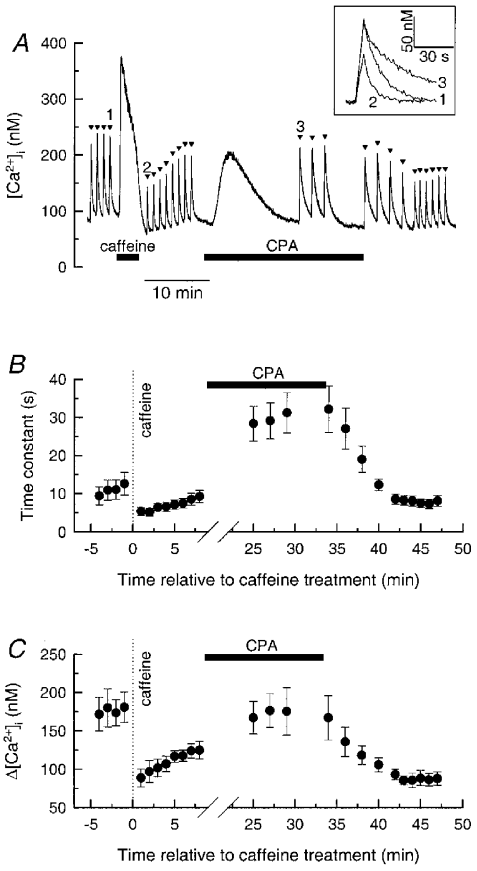

Figure 9. Action potential-induced $[Ca^{2+}]_i$ transients are modulated by the level of Ca^{2+} in ryanodine-sensitive Ca^{2+} stores

A, $[Ca^{2+}]_i$ transients were elicited by extracellular field stimulation (10 Hz, 4 s) in intact neurones loaded with indo-1 AM, as indicated by triangles above the $[Ca^{2+}]_i$ trace. Horizontal bars indicate application of 5 mM caffeine or 5 μM CPA. A comparison of corresponding $[Ca^{2+}]_i$ transients before (1) and after (2) caffeine application, or in the presence of CPA (3), is shown in the inset. The traces were offset along the $[Ca^{2+}]_i$ axes. In *B* and *C*, the changes in time constant that characterize the $[Ca^{2+}]_i$ recovery process (*B*) and the amplitude of the $[Ca^{2+}]_i$ elevation (*C*) for the experimental protocol described in *A* are analysed for 5 cells. Each point represents the mean ± S.E.M. of the corresponding parameter for the action potential-induced $[Ca^{2+}]_i$ transients. A monoexponential function was fitted to the $[Ca^{2+}]_i$ recovery process and the corresponding time constant was calculated by using a non-linear, least-squares curve fitting algorithm (Origin software). The data are plotted *versus* time, which was set to zero during the caffeine treatment (vertical dotted line).

5 ± 1 s ($n = 5$; Fig. 9*B*) and it decreased the amplitude of the response from 181 ± 20 to 89 ± 12 nM ($n = 5$; Fig. 9*C*). This was probably the consequence of the increased capability of the emptied stores to buffer cytosolic Ca^{2+}. Both parameters slowly recovered as the stores recharged during subsequent stimulation. Inhibition of the SERCAs with 5 μM CPA excluded Ca^{2+} stores from the buffering process and the resulting $[Ca^{2+}]_i$ transients increased in amplitude to 167 ± 21 nM ($n = 5$) and recovery slowed more than 3-fold ($\tau = 28 \pm 5$ s; $n = 5$) relative to the last $[Ca^{2+}]_i$ transient before CPA application ($\Delta[Ca^{2+}]_i = 125 \pm 11$ nM and $\tau = 9 \pm 2$ s; $n = 5$). The effect of CPA reversed completely in 5–8 min (Fig. 9). This is the first demonstration that intracellular stores contribute significantly to the amplitude and recovery kinetics of action potential-elicited $[Ca^{2+}]_i$ transients. These findings corroborate previous observations (Friel & Tsien, 1992; Usachev *et al.* 1993; Toescu, 1998) that depletion of ryanodine-sensitive Ca^{2+} stores enhances Ca^{2+} uptake into the stores.

Caffeine-induced $[Ca^{2+}]_i$ oscillations at resting membrane potential are controlled by store-regulated Ca^{2+} influx

We found that in 37% ($n = 134$) of large (28–34 μm) DRG neurones 5 mM caffeine initiated $[Ca^{2+}]_i$ oscillations (Fig. 10). These oscillations resulted from periodic Ca^{2+} release from and reuptake into ryanodine-sensitive stores (Lipscombe *et al.* 1988; Nohmi *et al.* 1992). The oscillations were maintained in neurones voltage clamped at −55 mV. However, they required a persistent Ca^{2+} influx, because addition of 2 mM Ni^{2+} to the buffer (Fig. 10*A*) or removal of extracellular Ca^{2+} (Fig. 10*B*) abolished the oscillations. The influx was not mediated by VOCCs because nimodipine (10 μM) and GsTX (1 μM) were without effect ($n = 4$). As shown in Fig. 10*B–D*, hyperpolarization from −55 to −80 mV increased significantly the frequency of oscillations from 0·6 ± 0·3 to 0·8 ± 0·3 min^{-1} ($n = 5$). This probably resulted from an increase in Ca^{2+} influx upon hyperpolarization and faster recharge of the Ca^{2+} stores, which subsequently accelerated the onset of the next $[Ca^{2+}]_i$ spike.

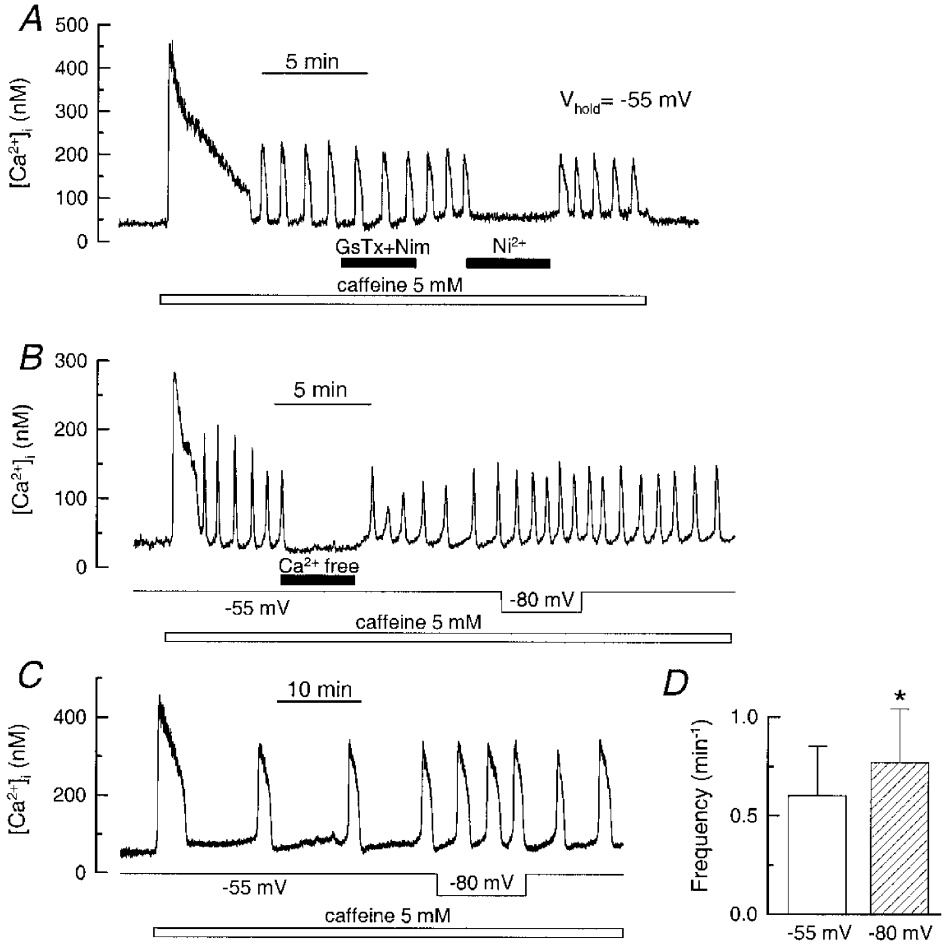

Figure 10. $[Ca^{2+}]_i$ oscillations are modulated by store-regulated Ca^{2+} influx in DRG neurones

A–C, $[Ca^{2+}]_i$ oscillations were induced by 5 mM caffeine in neurones clamped at −55 or −80 mV as indicated. The cells were loaded with 100 μM indo-1 through the patch pipette. Removal of extracellular Ca^{2+}, treatment with a combination of 1 μM GsTX and 10 μM nimodipine (GsTX + Nim) and application of 2 mM Ni^{2+} are indicated by horizontal bars below the $[Ca^{2+}]_i$ traces. In *D*, the frequency of $[Ca^{2+}]_i$ oscillations for membrane holding potentials of −55 and −80 mV are compared for 5 cells in experiments similar to those described in *B* and *C*. * $P < 0·05$; Student's paired *t* test.

DISCUSSION

In this report, we describe a Ca^{2+} influx pathway present in quiescent DRG neurones. The influx was not mediated by VOCCs, but rather shared certain features with capacitative Ca^{2+} influx. Hyperpolarization and depletion of ryanodine-sensitive Ca^{2+} stores enhanced the influx. This modest but persistent Ca^{2+} influx appeared to regulate the rate at which Ca^{2+} stores refilled with Ca^{2+}, and produced marked effects on store-regulated processes such as Ca^{2+} buffering and $[Ca^{2+}]_i$ oscillations.

Routes of Ca^{2+} entry in resting neurones

Neurones express multiple types of VOCCs (Bertolino & Llinás, 1992; Tsien *et al.* 1995). Upon excitation, Ca^{2+} enters the cell through these channels to initiate a number of processes including neurotransmitter release and transcription (Ghosh & Greenberg, 1995). This depolarization-induced Ca^{2+} influx also potentiates replenishment of intracellular Ca^{2+} stores, and thus depolarization is commonly used to refill Ca^{2+} stores in neurones (Usachev *et al.* 1993; Garaschuk *et al.* 1997). However, at the resting membrane potential, we found that VOCCs contributed little to Ca^{2+} influx or to refilling of Ca^{2+} stores in rat DRG neurones. Ca^{2+} influx was the rate-limiting step in the refilling of Ca^{2+} stores as indicated by the requirement for extracellular Ca^{2+}, its block by Ni^{2+} and the enhanced refilling produced by hyperpolarization. In spite of this exquisite sensitivity to factors that influence Ca^{2+} influx, pharmacological block of VOCCs with dihydropyridine drugs and Ca^{2+} channel toxins had no effect on the refilling of the stores, resting Ca^{2+} influx, or Mn^{2+} influx. These drugs were clearly effective under our recording conditions (Figs 2*B* and *C* and 3*B*). The action of dihydropyridines is voltage dependent; depolarization enhances their inhibitory effect on L-type channels. However, at concentrations above 100 nM these drugs are effective even at hyperpolarized membrane potentials (McCarthy & TanPiengco, 1992). Therefore, it is unlikely that a lack of effect of 10 μM nimodipine on the refilling process could be explained by its voltage dependence. In fact, short pre-treatment of DRG neurones with 10 μM nimodipine at the resting membrane potential completely abolished high-K^+-induced Ca^{2+} flux through L-type channels (Fig. 2*B*). Our observations are in agreement with the observation that dihydropyridines did not affect Ca^{2+} influx at rest in a neuronal cell line (Takemura *et al.* 1991), sympathetic (Lampe *et al.* 1995) or hippocampal neurones (Garaschuk *et al.* 1997) and are in contrast to another study that described a steady-state current mediated by dihydropyridine-sensitive channels in central neurones (Magee *et al.* 1996). This discrepancy might result from differences in dihydropyridine-sensitive (L-type) channels expressed in brain relative to peripheral tissues (Tsien *et al.* 1995) or the different methods used in these studies. The fact that VOCCs do not contribute to Ca^{2+} influx in DRG neurones at rest is in agreement with the voltage dependence of activation and inactivation for VOCCs in these cells (Fox *et al.* 1987). Despite the narrow 'window current' for T-type channels near the resting membrane potential (between −70 and −60 mV), their involvement is unlikely because hyperpolarization to −80 mV did not reduce, but instead enhanced, Ca^{2+} influx in resting DRG neurones (Figs 4 and 10). Similarly, the Na^+–Ca^{2+} exchanger, which could potentially carry Ca^{2+} into the cell in reverse mode (Miller, 1991), can also be excluded from consideration because hyperpolarization would favour Ca^{2+} extrusion from the cell. Thus, in this study we describe a novel route of Ca^{2+} influx into resting DRG neurones.

The Ca^{2+} influx described here shares a number of features with SOCCs found in non-excitable cells (Berridge, 1995; Parekh & Penner, 1997). Ca^{2+} influx was: (1) enhanced by Ca^{2+} store depletion, (2) enhanced by hyperpolarization, (3) blocked by Ni^{2+}, and (4) not sensitive to selective VOCC antagonists. The idea that SOCCs might participate in neuronal function is consistent with the high level of expression of mammalian homologues of the *Drosophila trp* gene in brain; TRP channels are believed to mediate capacitative Ca^{2+} entry (Mori *et al.* 1998; Philipp *et al.* 1998). Ca^{2+} influx induced by store depletion was most easily detected as a sustained $[Ca^{2+}]_i$ plateau phase during the caffeine-induced response (Fig. 5), and as a $[Ca^{2+}]_i$ overshoot following the switch from Ca^{2+}-free to 2 mM Ca^{2+}-containing media (Fig. 6). In the latter case, a similar result might occur if Ca^{2+} buffering mechanisms were downregulated during the Ca^{2+}-free treatment, but this was not the case because the overshoot was dependent on depletion of the store by caffeine (Fig. 6), a treatment that actually enhanced buffering of electrically induced $[Ca^{2+}]_i$ increases (Fig. 9). Furthermore, the influx of Mn^{2+}, a cation that permeates SOCCs (Jacob, 1990), was enhanced by approximately 2-fold after depletion of the stores. The recovery of the overshoot was approximately 10-fold slower than that of electrically induced $[Ca^{2+}]_i$ transients suggesting that termination of the overshoot was determined by inactivation of Ca^{2+} influx rather than by Ca^{2+} buffering processes. Once activation of SOCCs is initiated by depletion of the stores, these channels inactivate as the consequence of refilling of the store (Jacob, 1990; Zweifach & Lewis, 1995). In DRG neurones, the time course for refilling the stores with Ca^{2+} was characterized by virtually the same kinetics ($\tau = 137$ s) as the recovery from the overshoot ($\tau = 123$ s), suggesting that inactivation of Ca^{2+} influx in these cells was also controlled by replenishment of intracellular Ca^{2+} stores.

Store-operated Ca^{2+} influx in non-excitable cells has been mainly attributed to depletion of inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores (Parekh & Penner, 1997). Here we demonstrate a similar link between depletion of ryanodine-sensitive Ca^{2+} stores and Ca^{2+} influx. Both types of store are associated with the endoplasmic reticulum and there is a high level of structural and functional similarity between IP_3 and ryanodine receptors (Pozzan *et al.* 1994). The fact that Ca^{2+} influx can be activated by depletion of Ca^{2+} stores with SERCA inhibitors (Thomas & Hanley, 1994) indicates that formation of IP_3 is not required for this activation.

Therefore, it is plausible that depletion of ryanodine-sensitive stores might initiate mechanisms similar to those proposed for the IP_3-sensitive stores (Berridge, 1995; Parekh & Penner, 1997). Indeed, activation of Ca^{2+} influx by depletion of ryanodine-sensitive Ca^{2+} stores has been reported recently in PC12 cells (Clementi *et al.* 1992; Bennett *et al.* 1998). This influx was similar to that evoked by IP_3 or the SERCA inhibitor thapsigargin in the same cells, suggesting that different intracellular Ca^{2+} stores may utilize common pathways to induce capacitative Ca^{2+} entry.

Physiological role of the store-regulated Ca^{2+} influx in neurones

While voltage-gated Ca^{2+} channels are the major pathway for Ca^{2+} influx during excitation in neurones, the novel mechanism of Ca^{2+} influx described in this study may be important between periods of electrical activity. We estimate that the electrical current corresponding to this influx, based on the maximal rate of $[Ca^{2+}]_i$ rise during the $[Ca^{2+}]_i$ overshoot (Fig. 6), is approximately 3 orders of magnitude smaller than the Ca^{2+} current activated during an action potential (Fox *et al.* 1987; Thayer & Miller, 1990). However, because this influx persists at resting membrane potential, in 1–2 s it carries as much Ca^{2+} as that carried during an action potential. The sustained Ca^{2+} influx elicited by store depletion in sympathetic neurones protected these cells from apoptosis induced by nerve growth factor deprivation (Lampe *et al.* 1995).

The Ca^{2+} influx described here served to replenish intracellular Ca^{2+} stores. The Ca^{2+} level within the stores is important for several reasons. The stores participate in removal of Ca^{2+} from the cytosol during cell excitation. We found that the efficiency of this transport was significantly increased when the stores were depleted. Depletion of the stores with 5 mM caffeine resulted in a more than 2-fold decrease in the amplitude and duration of electrically induced $[Ca^{2+}]_i$ responses (Fig. 9), both of which recovered as the stores refilled. Caffeine (10 mM) reduced Ca^{2+} currents by approximately 25% in sympathetic neurones, although the current recovered within a few seconds after removal of the drug (Lipscombe *et al.* 1988). An effect of caffeine on VOCCs was unlikely to influence the experiments described here because the first post-caffeine electrical stimulation was applied 60 s after the drug was completely washed from the bath (Fig. 9*A*), as confirmed by recovery from the caffeine-induced quench of indo-1 fluorescence (O'Neill *et al.* 1990). Thus, our observations indicate that the Ca^{2+} level within the store determines the size and duration of the $[Ca^{2+}]_i$ transients elicited by a given pattern of electrical activity, which in turn would control triggering of various Ca^{2+}-dependent cell functions (Ghosh & Greenberg, 1995). Chronic depletion of Ca^{2+} stores has been shown to disturb gene expression by inhibiting molecular transport across the nuclear envelope (Perez-Terzik *et al.* 1997) and to block protein synthesis (Thomas & Hanley, 1994).

Ca^{2+} influx was required to maintain caffeine-induced $[Ca^{2+}]_i$ oscillations at resting membrane potential in neurones (Fig. 10). Store-regulated Ca^{2+} entry enabled changes in membrane potential to tune the frequency of $[Ca^{2+}]_i$ oscillations. The exact role of $[Ca^{2+}]_i$ oscillations in neurones is not clear, although some observations suggest that certain patterns of periodic $[Ca^{2+}]_i$ spikes are important for regulation of gene expression (Itoh *et al.* 1995) and cell growth and differentiation (Gu & Spitzer, 1995; Gomez *et al.* 1995).

We have shown that mammalian sensory neurones possess a distinct Ca^{2+} entry pathway that is active at rest and enhanced by depletion of Ca^{2+} stores. This observation is consistent with studies in non-excitable cells that describe specialized channels that are somehow activated by store depletion and play an important role in refilling the store with Ca^{2+} and maintaining sustained Ca^{2+} influx. Why neurones, with their extensive complement of voltage- and receptor-operated Ca^{2+} channels, need yet another Ca^{2+} entry pathway is not entirely clear. However, the pathway is present and appears to maintain the Ca^{2+} balance between intracellular and extracellular pools in DRG neurones at resting membrane potential.

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Corresponding author

S. A. Thayer: Department of Pharmacology, University of Minnesota Medical School, 3-249 Millard Hall, 435 Delaware Street SE, Minneapolis, MN 55455, USA.

Email: thayer@med.umn.edu