Release and sequestration of calcium by ryanodine-sensitive stores in rat hippocampal neurones

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1. The properties of ryanodine-sensitive Ca^{2+} stores in CA1 pyramidal cells were investigated in rat hippocampal slices by using whole-cell patch-clamp recordings combined with fura-2-based fluorometric digital imaging of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$).

2. Brief pressure applications of caffeine onto the somata of pyramidal cells caused large transient increases in $[Ca^{2+}]_i$ (Ca^{2+} transients) of 50–600 nM above baseline.

3. The Ca^{2+} transients evoked by caffeine at −60 mV were not associated with an inward current, persisted after blocking voltage-activated Ca^{2+} currents and were completely blocked by bath-applied ryanodine. Similar transients were also evoked at +60 mV. Thus, these transients reflect Ca^{2+} release from intracellular ryanodine-sensitive Ca^{2+} stores.

4. The Ca^{2+} transients evoked by closely spaced caffeine pulses rapidly decreased in amplitude, indicating progressive depletion of the Ca^{2+} stores. The amplitude of the Ca^{2+} transients recovered spontaneously with an exponential time constant of 59 s. Recovery was accelerated by depolarization-induced elevations in $[Ca^{2+}]_i$ and blocked by cyclopiazonic acid (CPA) and thapsigargin, indicating that store refilling is mediated by endoplasmic reticulum Ca^{2+}-ATPases.

5. Even without prior store depletion the caffeine-induced Ca^{2+} transients disappeared after 6 min exposure to CPA, suggesting that ryanodine-sensitive Ca^{2+} stores are maintained at rest by continuous Ca^{2+} sequestration.

6. Caffeine-depleted Ca^{2+} stores did not refill in Ca^{2+}-free saline, suggesting that the refilling of the stores depends upon Ca^{2+} influx through a 'capacitative-like' transmembrane influx pathway operating at resting membrane potential. The refilling of the stores was also blocked by Ni^{2+} and gallopamil (D600).

7. Elevations of basal $[Ca^{2+}]_i$ produced by bath-applied KCl markedly potentiated (up to 6-fold) the caffeine-induced Ca^{2+} transients. The degree of potentiation was positively related to the increase in basal $[Ca^{2+}]_i$. The Ca^{2+} transients remained potentiated up to 9 min after reversing the KCl-induced $[Ca^{2+}]_i$ increase. Thus, the ryanodine-sensitive Ca^{2+} stores can 'overcharge' when challenged with an increase in $[Ca^{2+}]_i$ and slowly discharge excess Ca^{2+} after basal $[Ca^{2+}]_i$ returns to its resting level.

8. Pressure applications of caffeine onto pyramidal cell dendrites evoked local Ca^{2+} transients similar to those separately evoked in the respective somata. Thus, dendritic ryanodine-sensitive Ca^{2+} stores are also loaded at rest and can function as independent compartments.

9. In conclusion, the ryanodine-sensitive Ca^{2+} stores in hippocampal pyramidal neurones contain a releasable pool of Ca^{2+} that is maintained by a Ca^{2+} entry pathway active at subthreshold membrane potentials. Ca^{2+} entry through voltage-gated Ca^{2+} channels transiently overcharges the stores. Thus, by acting as powerful buffers at rest and as regulated sources during activity, Ca^{2+} stores may control the waveform of physiological Ca^{2+} signals in CA1 hippocampal pyramidal neurones.

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The ryanodine-sensitive intracellular Ca^{2+} stores are known to play a major role in excitation–contraction coupling in skeletal and cardiac muscle (see McPherson & Campbell, 1993; Sutko & Airey, 1996). Much less is known about the function of these stores in central neurones, despite the fact that all known members of the ryanodine receptor (RyR) family, namely, skeletal muscle type (type I), cardiac muscle type (type II) and brain type (type III), are abundantly expressed in the central nervous system (Furuichi, Furutama, Hakamata, Nakai, Takeshima & Mikoshiba, 1994).

According to one hypothesis, RyRs in peripheral and central neurones amplify and prolong incoming Ca^{2+} signals via Ca^{2+}-induced Ca^{2+} release (CICR) from the ryanodine-sensitive Ca^{2+} stores (Holliday, Adams, Sejnowski & Spitzer, 1991; Hua, Nohmi & Kuba, 1993; Llano, DiPolo & Marty, 1994; Kano, Garaschuk, Verkhratsky & Konnerth, 1995), thus sharing functional properties with cardiac muscle RyRs (Fabiato, 1983; Nabauer, Callewaert, Cleemann & Morad, 1989). Accordingly, tetanic synaptic stimulation of CA1 pyramidal cells in a slice preparation reportedly induced Ca^{2+} release from dendritic ryanodine-sensitive Ca^{2+} stores (Alford, Frenguelli, Schofield & Collingridge, 1993). A RyR-mediated CICR process, preferentially localized to dendritic spines, may boost the magnitude and duration of the Ca^{2+} signals required for induction of long-term potentiation of synaptic efficacy in the hippocampus (Schiegg, Gerstner, Ritz & van Hemmen, 1995; Wang, Wu, Rowan & Anwyl, 1996). However, ryanodine-sensitive Ca^{2+} stores in different types of cultured rat central neurones (including CA1 hippocampal neurones) were reported to be empty at rest and to accumulate Ca^{2+} only after its entry via voltage-gated Ca^{2+} channels in the plasmalemma (Brorson, Bleakman, Gibbons & Miller, 1991; Shmigol, Kirischuk, Kostyuk & Verkhratsky, 1994).

According to another hypothesis (see Simpson, Challiss & Nahorski, 1995), neuronal ryanodine-sensitive Ca^{2+} stores may act as a buffering system for intracellular Ca^{2+} ions (Ca_i^{2+}). Firstly, KCl-induced increases in $[Ca^{2+}]_i$ in bullfrog sympathetic neurones, were substantially attenuated after depletion of ryanodine-sensitive Ca^{2+} stores by prolonged caffeine application (Friel & Tsien, 1992). Secondly, blockers of sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), prolonged the depolarization-induced increases in dendritic $[Ca^{2+}]_i$ in rat neocortical layer V pyramidal neurones in slices (Markram, Helm & Sakmann, 1995). Thus, sequestration of cytosolic Ca^{2+} by intracellular Ca^{2+} stores may contribute substantially to Ca_i^{2+} clearance in peripheral and central neurones.

Using fluorometric digital imaging of $[Ca^{2+}]_i$ combined with whole-cell patch-clamp recordings in rat hippocampal slices, we have investigated the dynamic properties of the ryanodine-sensitive Ca^{2+} stores in CA1 pyramidal cells. These neurones express the three types of RyRs and, compared with other central neurones, have the highest level of the brain-type RyRs (Furuichi *et al.* 1994). Moreover, in these neurones the RyRs are expressed in the axon, soma and dendrites, including the dendritic spines (Sharp, McPherson, Dawson, Aoki, Campbell & Snyder, 1993) and thus occupy strategically important positions for synaptic signalling and integration.

METHODS

Slice preparation and solutions

Hippocampal slices (300 μm thick) were prepared from brains of young (9–18 day old) Wistar rats as described previously (Edwards, Konnerth, Sakmann & Takahashi, 1989; Garaschuk, Schneggenburger, Schirra, Tempia & Konnerth, 1996). Briefly, rats were decapitated and brain hemispheres were rapidly isolated and placed in an ice-cold bicarbonate-buffered standard saline (see below for composition). Slices were transferred to a storage chamber containing standard saline bubbled with 95% O_2–5% CO_2.

The composition of the standard saline was (mM): 125 NaCl, 2·5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1·25 NaH_2PO_4, 26 $NaHCO_3$ and 20 glucose (pH 7·4 when bubbled continuously with 95% O_2–5% CO_2). To block voltage-gated sodium channels 0·5 μM tetrodotoxin (TTX) was added to the standard saline in all experiments. In Ca^{2+}-free saline the $CaCl_2$ was replaced with 1 mM $MgCl_2$ and 0·1 mM EGTA was added.

The standard pipette (intracellular) solution contained (mM): 130 CsCl, 20 tetraethylammonium chloride (TEACl), 4 Mg-ATP, 0·2 Na-GTP, 100–200 μM fura-2 pentapotassium salt and 10 Hepes (pH 7·3, adjusted with CsOH). In several experiments 140 mM KCl and 10 mM NaCl were used instead of CsCl and TEACl, as indicated.

All chemicals were purchased from Sigma. D600 (gallopamil) was a gift from Knoll (Ludwigshafen, Germany).

Electrophysiological recordings and drug application

Experiments were performed at room temperature (22–24 °C) in an experimental set-up equipped with an upright microscope (Axioscope FS; for details see also Eilers, Schneggenburger & Konnerth, 1995). CA1 pyramidal neurones in hippocampal slices were visually identified according to their characteristic shape and their location within the cell body layer (Konnerth, 1990; Garaschuk *et al.* 1996). Before establishing the whole-cell recording configuration the membrane surface was cleaned, if necessary, using procedures described previously (Edwards *et al.* 1989). Patch pipettes were made of borosilicate glass (Hilgenberg GmbH, Malsfeld, Germany) and coated with a silicon-based resin (General Electrics, Ruesselsheim, Germany). They had a resistance of 2–3 MΩ when filled with the intracellular solution. Membrane currents were recorded with the EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany). The holding potential was set, if not otherwise indicated, at −60 mV. Data acquisition and pulse protocols for controlling drug application (see below) were performed using 'Pulse' software (HEKA). The pipette series resistance was less than 10–12 MΩ. In some experiments it was compensated up to 60% following standard procedures.

Caffeine was pressure applied using a Picospritzer II (General Valve, Fairfield, NJ, USA) from a fine pipette (6–12 MΩ) filled with standard saline in which 10 or 20 mM NaCl was substituted with 20 or 40 mM caffeine, respectively. The tip of the caffeine application

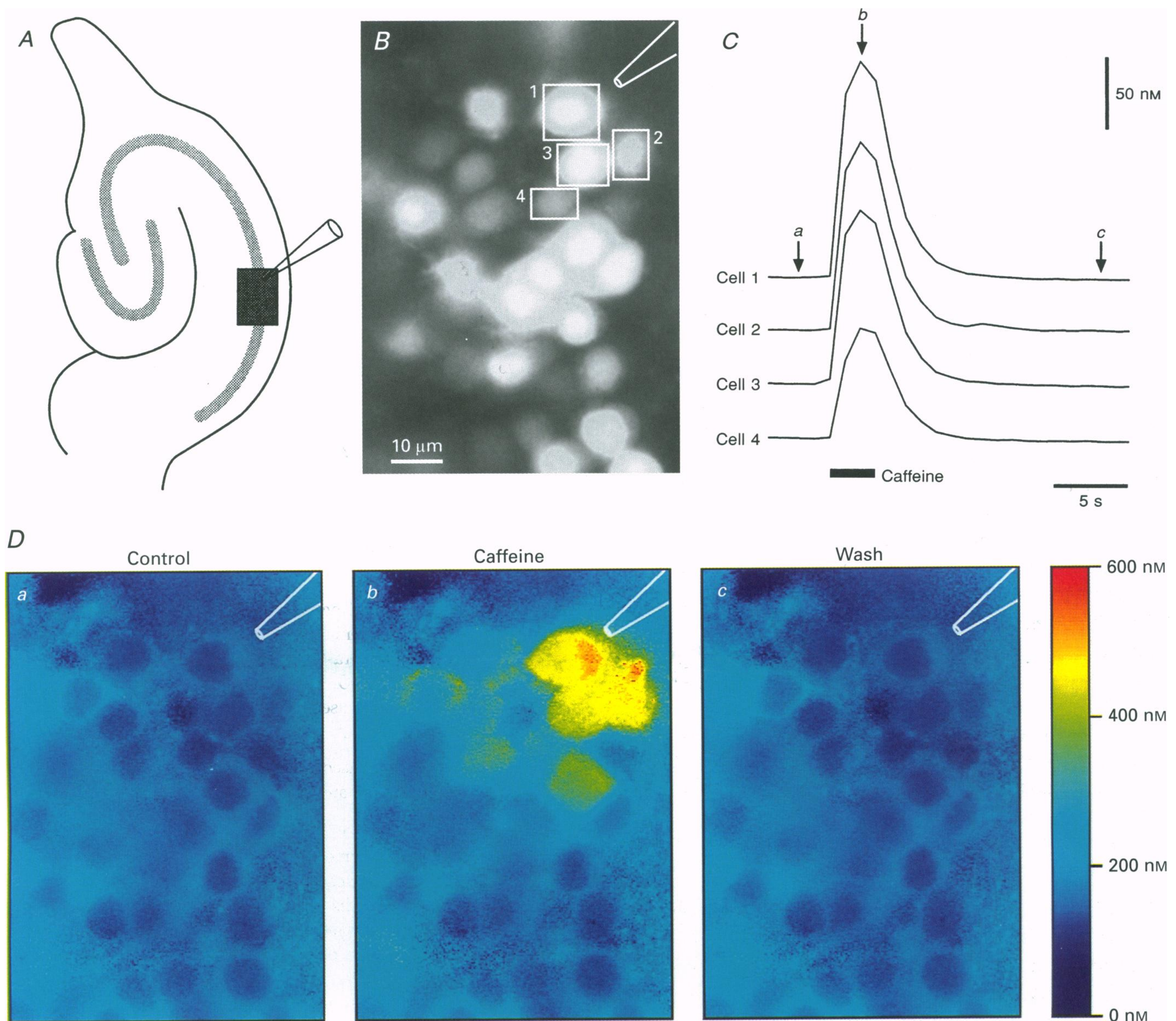

Figure 1. Pressure-applied caffeine induces a transient increase in $[Ca^{2+}]_i$ in CA1 pyramidal neurones

A, the experimental arrangement for pressure application of caffeine and fluorometric $[Ca^{2+}]_i$ measurements in CA1 hippocampal pyramidal neurones loaded with fura-2 AM. The position of the application pipette with respect to the field of view (shaded box) and to the CA1 pyramidal layer is shown. *B*, digital fluorescence image of the slice taken at 380 nm excitation wavelength. Regions of interest incorporating the somata of four neurones in which fluorometric $[Ca^{2+}]_i$ measurements were made are marked 1–4. The position of the caffeine-containing application pipette is schematically indicated in the upper right corner. Here and in the experiments illustrated in the following figures the application pipette contained 40 mM caffeine, if not otherwise indicated. *C*, fluorometric measurements of $[Ca^{2+}]_i$ in the four selected regions, depicting the simultaneous increase in $[Ca^{2+}]_i$ evoked by caffeine application (3 s; bar). Downward arrows in *C* marked with *a*, *b* and *c* indicate the time at which the fluorescence images in *D* were taken. *D*, pseudocolour fluorescence images taken before (*a*, Control), during (*b*, Caffeine) and after (*c*, Wash) caffeine application. Scale bar in *B* also applies to *D*.

pipette was placed near (~5 μm) a deliberately chosen primary target neurone (cell 1 in Fig. 1*B*) and, for a given caffeine concentration in the pipette, the shortest pulse duration (2–8 s) evoking a reproducible response was selected. We estimate that the true concentration of caffeine reaching this cell was less than 10 mM. To obtain 80 mM KCl-containing saline, 80 mM NaCl was substituted for KCl in the standard perfusion saline.

Fluorometric Ca^{2+} measurements

Fluorometric Ca^{2+} measurements were performed with fura-2 (pentapotassium salt; Molecular Probes) added to the pipette solution at a concentration of 100–200 μM and loaded into the cell via the patch pipette. Alternatively, cells were loaded with the acetoxymethyl ester of fura-2 (fura-2 AM; Molecular Probes). For this purpose slices were incubated for 10 min in the oxygenated saline containing fura-2 AM (10–15 μM) at 36 °C. A reasonably good loading of neuronal somata located in the top layers of the slice was routinely achieved.

Fluorometric measurements were done using a variable-scan digital imaging system (T.I.L.L. Photonics GmbH, München, Germany) with 'Image-8' software (University of Saarland, Homburg, Germany). Digital fluorescence images of the slice were obtained using paired exposures to 360 and 380 nm excitation wavelength. Following the establishment of the whole-cell configuration, the loading of the cell with fura-2 was monitored as a gradual increase in the Ca^{2+}-independent fluorescence signal. Measurements were started only after equilibration of the fura-2 concentration between the pipette and all cellular compartments of interest (e.g. soma and dendrites), as indicated by stable maximal intensity signals at 360 nm excitation wavelength. In general, the time taken to reach this equilibrium was about 10 min.

Routinely, fluorescence values representing spatial averages from up to three defined pixel regions were transferred on-line through a serial port to another personal computer and displayed there as a function of time together with the whole-cell current recording (Eilers *et al.* 1995). In most cases the regions of interest were placed on somata of different pyramidal cells. Thus, each experiment provided simultaneous measurements from up to three different neurones. The background fluorescence was measured in a separate pixel region near the cell and used for an on-line correction of the cellular fluorescence signals. The calibration constants K_{eff} (effective binding constant), R_{min} (fluorescence ratio at zero Ca^{2+}) and R_{max} (limiting fluorescence ratio at high Ca^{2+}) were obtained from *in vivo* calibration experiments (Neher, 1989; Eilers *et al.* 1995). Typical calibration parameters found for K_{eff}, R_{min} and R_{max} were 1048, 0·67 and 4·2 nM, respectively. In some instances (see Fig. 1), consecutive paired exposures to 360 and 380 nm were used to construct background-corrected digital fluorescence images. They were displayed on-line in a pseudocolour mode on a monitor and stored on hard disk for later analysis.

Data are given as means ± standard deviation (S.D.).

RESULTS

Resting basal $[Ca^{2+}]_i$ was 49·5 ± 14·4 nM ($n = 76$ cells from 33 slices) in pyramidal cells loaded with fura-2 AM and 42·5 ± 19·7 nM ($n = 52$ cells from 42 slices) in whole-cell clamped pyramidal cells loaded with fura-2 via the recording patch pipette. The difference between the mean values was not significant ($P > 0·25$, Student's two-tailed t test).

Caffeine-induced increases in $[Ca^{2+}]_i$ in somata of pyramidal cells

Figure 1 illustrates fluorometric measurements obtained from neurones in the CA1 pyramidal cell layer (Fig. 1*A*), which were loaded with fura-2 AM (Fig. 1*B*). Brief (3 s) pressure application of caffeine rapidly evoked a marked increase in $[Ca^{2+}]_i$ (Ca^{2+} transient) in the neurones immediately adjacent

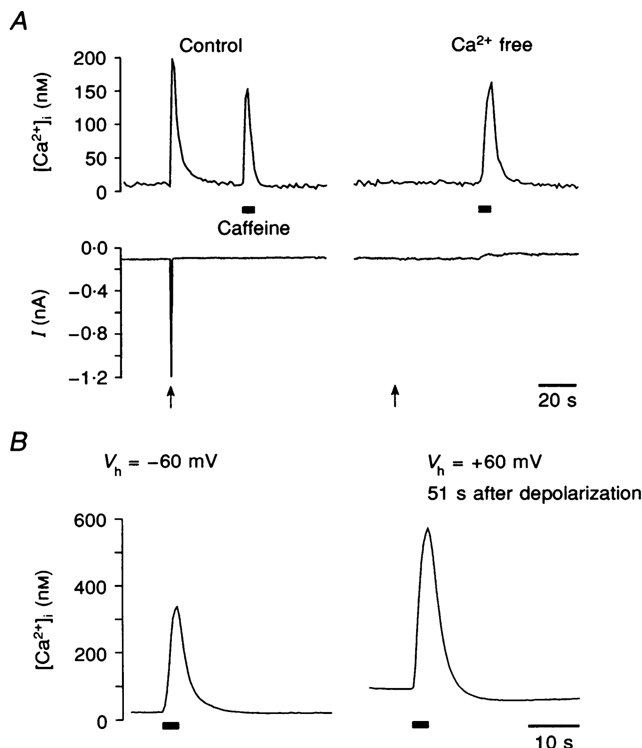

Figure 2. Caffeine induces Ca^{2+} release from internal stores

A, simultaneous recordings of somatic $[Ca^{2+}]_i$ (top traces) and membrane current (bottom traces) in a whole-cell clamped CA1 pyramidal cell filled with fura-2. In this and in the following figures each upward arrow indicates a 200 ms depolarizing voltage pulse from −60 to 0 mV, if not otherwise stated. In standard saline (control; left panel), a depolarizing pulse evoked a large Ca^{2+} current associated with a transient rise in $[Ca^{2+}]_i$. After 4 min of perfusing the slice with Ca^{2+}-free saline (right panel), the depolarizing pulse produced neither a Ca^{2+} current nor a $[Ca^{2+}]_i$ increase (right panel). In contrast, caffeine application (6 s; bar) induced similar Ca^{2+} transients in standard and in Ca^{2+}-free saline solutions (amplitudes of 148 and 145 nM, respectively). *B*, in another cell, application of caffeine (3 s; bar) evoked Ca^{2+} transients both at −60 mV (left panel) and +60 mV (right panel) holding potentials (V_h). The latter response was evoked 51 s after stepping to +60 mV. Note that at +60 mV basal $[Ca^{2+}]_i$ increased, most probably due to Ca^{2+} entry through voltage-gated Ca^{2+} channels activated by stepping to +60 mV holding potential. The increase in basal $[Ca^{2+}]_i$ was associated with an increase in amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient.

to the application pipette (Fig. 1*D*). The spatially averaged somatic $[Ca^{2+}]_i$ values obtained from four neurones in the field of view (delineated in Fig. 1*B*) were plotted as a function of time in Fig. 1*C*. In these cells, numbered 1–4, caffeine increased $[Ca^{2+}]_i$ by 156, 136, 127 and 80 nM, respectively. These peak values were attained during caffeine application and prolongation of the caffeine pulse did not cause a further rise in $[Ca^{2+}]_i$ (not shown). This suggests that the Ca^{2+} transients were the maximal responses to the caffeine concentrations that stimulated the cells. Expectedly, neurones that were closer to the application pipette (e.g. cell 1 in Fig. 1) displayed larger and faster caffeine-evoked Ca^{2+} transients than those located downstream (e.g. cell 4 in Fig. 1).

The amplitudes of caffeine-induced Ca^{2+} transients in 105 pyramidal neurones located in the immediate vicinity of the caffeine application pipette ranged from 50 to 600 nM above basal $[Ca^{2+}]_i$, with few exceptions exceeding this range (mean 253 ± 269, $n = 105$ cells from 85 slices). The large scatter in amplitudes of these Ca^{2+} transients probably reflects the variation in the concentrations of applied caffeine that actually stimulated the cells (compare cell 1 and cell 4 in Fig. 1).

Despite these differences in amplitude, the caffeine-evoked Ca^{2+} transients were highly robust and reproducible in most cells tested. In control experiments we found that only 11 out of 133 cells ($n = 9$ slices), which experienced caffeine concentration sufficient to evoke Ca^{2+} transients in surrounding cells, failed to respond to caffeine applications (not shown). One of these eleven cells became responsive after increasing the duration of application from 2·5 to 3 s suggesting that slightly higher concentrations of caffeine were necessary to stimulate these cells.

Caffeine induces Ca^{2+} release from internal stores

To identify the origin of the caffeine-induced Ca^{2+} transients we first examined whether these signals are associated with influx of extracellular Ca^{2+} ions. We tested this question in whole-cell clamped pyramidal cells in conditions which isolated Ca^{2+} currents (see Methods). As shown in Fig. 2*A* (left panel), a 200 ms depolarizing pulse from −60 to 0 mV evoked an inward current and a large increase in $[Ca^{2+}]_i$. By contrast, the caffeine-induced Ca^{2+} transient was not associated with any inward current. Perfusing the slice with nominally Ca^{2+}-free saline for about 4 min completely blocked the depolarization-induced inward current and the associated Ca^{2+} transient, while the caffeine-induced Ca^{2+} transient was preserved. Similar observations were made in seven experiments, supporting the notion that the caffeine-induced Ca^{2+} transients reflect the release of Ca^{2+} from internal stores.

Another test for the internal origin of these Ca^{2+} transients is illustrated in Fig. 2*B*. The pyramidal cells were held at the approximate Ca^{2+} equilibrium potential (+60 mV) to block transmembrane Ca^{2+} influx. Application of caffeine induced Ca^{2+} transients in all cases ($n = 6$ neurones) at this potential. Their amplitudes exceeded those of the transients

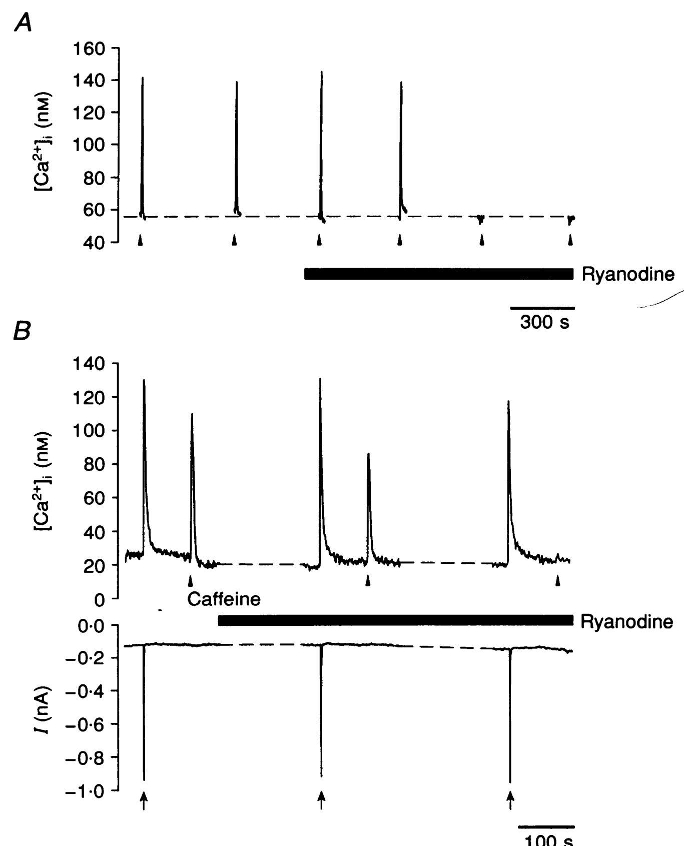

Figure 3. Ryanodine blocks caffeine-induced Ca^{2+} transients

A, continuous fluorometric $[Ca^{2+}]_i$ recording in an intact pyramidal cell loaded with fura-2 AM. In this and in the following figures brief caffeine applications are marked with arrowheads if not otherwise indicated. The caffeine-induced Ca^{2+} transients were abolished after 20 μM ryanodine (bar) was added to the saline. *B*, continuous recordings of $[Ca^{2+}]_i$ (upper trace) and membrane current recordings (lower trace) performed simultaneously in a whole-cell clamped pyramidal neurone filled with fura-2. Bath application of 10 μM ryanodine (bar) produced a progressive block of the caffeine-induced Ca^{2+} signals without significantly affecting the depolarization-evoked Ca^{2+} currents and associated rise in $[Ca^{2+}]_i$.

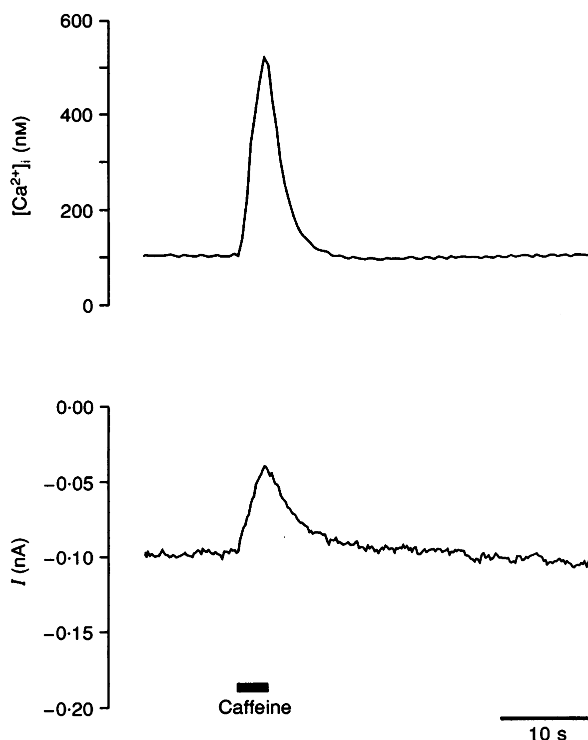

Figure 4. Caffeine-induced Ca^{2+} transient is associated with an outward K^+ current

Simultaneous $[Ca^{2+}]_i$ (upper trace) and membrane current (lower trace) recordings in a whole-cell clamped pyramidal cell. In this experiment the pipette solution contained 140 mM KCl and 10 mM NaCl instead of CsCl and TEACl. The thick bar indicates the time of caffeine application (3 s). The caffeine application induced a Ca^{2+} transient and an outward current, presumably a K^+ current activated by cytosolic Ca^{2+}.

recorded at resting membrane potential (Fig. 2*B*). This increase in amplitude is related to the increased level of $[Ca^{2+}]_i$ following Ca^{2+} entry through voltage-activated Ca^{2+} channels when stepping from −60 to +60 mV (see below for further explanation).

Ryanodine sensitivity of caffeine-induced Ca^{2+} release

The effect of ryanodine (20 μM) on caffeine-induced Ca^{2+} transients was examined in intact pyramidal cells loaded with fura-2 AM ($n = 19$). At this concentration, ryanodine was shown to bind to RyR channels and 'lock' them in an

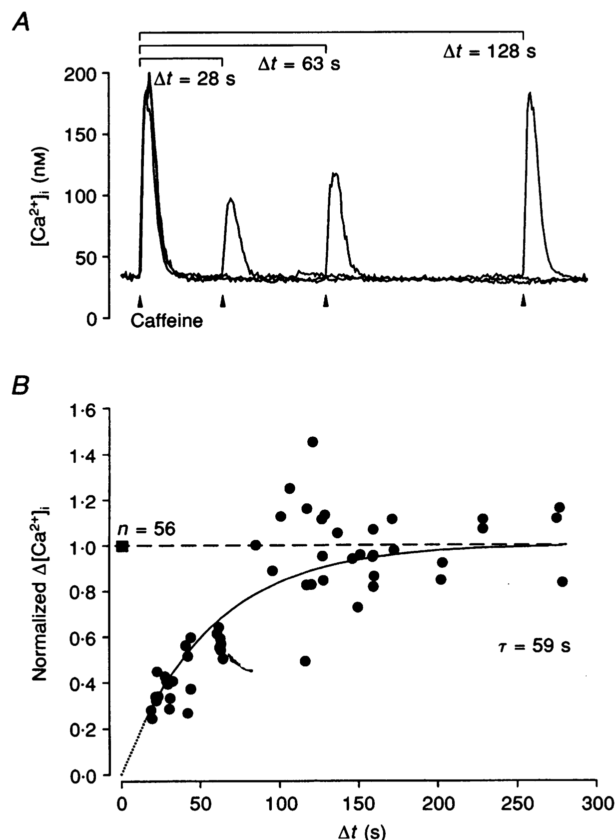

Figure 5. Depletion and spontaneous refilling of ryanodine-sensitive stores

A, somatic $[Ca^{2+}]_i$ recording in a pyramidal cell loaded with fura-2 AM. Ca^{2+} transients were evoked by two identical caffeine pulses (4 s) applied sequentially at different intervals. The three superimposed recordings of the responses to paired-pulse stimulation were aligned according to the conditioning Ca^{2+} transient. The interstimulus interval in each recording is indicated. *B*, pooled data obtained from fifty-six paired caffeine applications in twenty-nine neurones. Normalized amplitudes of Ca^{2+} transients were plotted against the time interval between conditioning and test caffeine pulses. The data points were fitted using the non-linear least-squares fit approach with a single exponential function (continuous line), yielding a time constant of 59 s for the spontaneous refilling of the caffeine-sensitive Ca^{2+} stores. The fit was done using an option provided by the data analysis program (Igor, Wave Metrics, Lake Oswego, OR, USA). Coefficients of the fit were: 1·07 ± 0·06, −1·12 ± 0·11 and 0·017 ± 0·004. The dashed line represents extrapolation of the fit to an interpulse interval of $\Delta t = 0$.

open subconducting state, leading to depletion of the ryanodine-sensitive Ca^{2+} stores (Rousseau, Smith & Meissner, 1987; Bezprozvanny, Watras & Ehrlich, 1991). As illustrated in Fig. 3*A*, the drug completely abolished the caffeine-induced Ca^{2+} transients in less than 10 min in all cells tested. Further, the effects of ryanodine on depolarization- *versus* caffeine-induced Ca^{2+} transients were compared in patch-clamped neurones loaded with fura-2 ($n = 2$). As shown in Fig. 3*B*, bath application of ryanodine completely abolished the caffeine-induced Ca^{2+} transients within 8 min, but not those evoked by depolarizing voltage pulses.

Taken together the data indicate that caffeine releases Ca^{2+} from ryanodine-sensitive Ca^{2+} stores in CA1 pyramidal cells. The fact that in virtually all pyramidal cells examined, Ca^{2+} transients were readily induced by caffeine prior to activation of any voltage-gated Ca^{2+} currents (e.g. Fig. 3*A*), indicates that even at rest the stores contain a releasable pool of Ca^{2+} ions.

Caffeine-induced Ca^{2+} release evokes a K^+ current

In pyramidal neurones dialysed with an intracellular solution containing K^+ as the main cation, the caffeine-induced Ca^{2+} transients were accompanied by outward membrane currents (Fig. 4; $n = 4$). The onset and peak of these outward currents were coincident with those of the associated Ca^{2+} transients. Such outward currents were never seen when the standard Cs^+-containing intracellular solution was used (e.g. Figs 2*A*, 3*B* and 14*A*). These observations demonstrate that Ca^{2+} release from ryanodine-sensitive stores increases $[Ca^{2+}]_i$ near the plasma membrane to a level that is sufficient to activate Ca^{2+}-activated K^+ channels.

Depletion and spontaneous refilling of ryanodine-sensitive Ca^{2+} stores

During a paired-pulse caffeine-application protocol, when caffeine was applied again shortly after a conditioning application, the evoked Ca^{2+} transient was diminished in amplitude (Fig. 5*A*). Recovery of the Ca^{2+} transient occurred spontaneously and gradually over a course of 2 min. The pooled data obtained from fifty-six paired-pulse caffeine applications to twenty-nine pyramidal cells is plotted in Fig. 5*B*. Fitting a single exponential function to these data (Fig. 5*B*, continuous line) yielded a recovery time constant of 59 s. Extrapolating the fitted function to an interpulse interval of $\Delta t = 0$ (Fig. 5*B*, dashed line) yielded a Ca^{2+} transient of zero amplitude.

A likely explanation for these observations is that the conditioning caffeine pulse empties the ryanodine-sensitive Ca^{2+} stores almost completely. After caffeine application the ryanodine-sensitive Ca^{2+} stores refill spontaneously, albeit slowly. Consistent with this notion, multiple closely spaced (e.g. 0·1 Hz) applications of caffeine elicited progressively smaller Ca^{2+} transients (Fig. 6*A*). Apparent depletion of internal Ca^{2+} stores was seen in all cells exposed to repetitive caffeine applications (46 intact and 9 patch-clamped pyramidal cells).

It may be argued that when exposed to a high caffeine concentration or to repeated caffeine applications, the Ca^{2+} release mechanism undergoes a form of desensitization, which renders it unresponsive to caffeine for some time. To distinguish between store depletion and desensitization, the experiment described in Fig. 6 was performed in sixteen

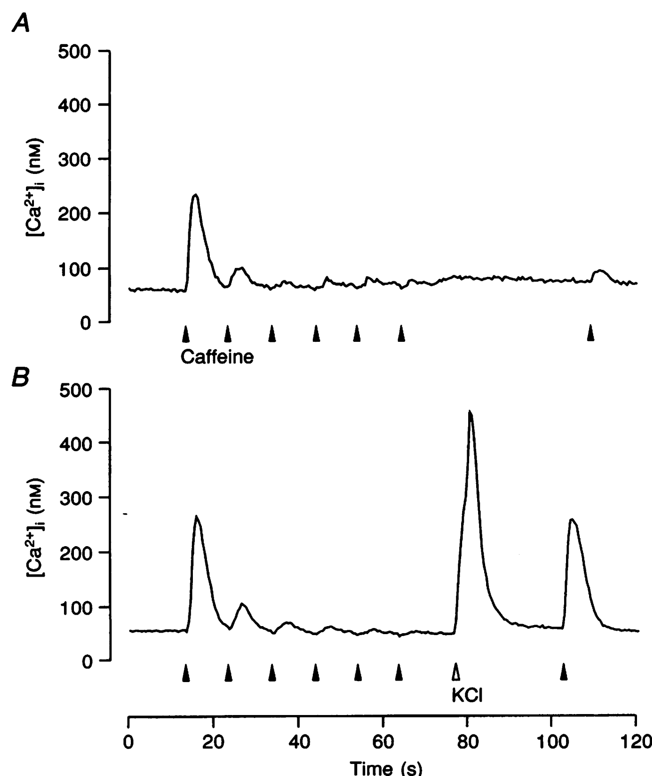

Figure 6. Depolarization-induced refilling of ryanodine-sensitive Ca^{2+} stores

Recordings of somatic $[Ca^{2+}]_i$ in an intact pyramidal cell loaded with fura-2 AM. Six consecutive caffeine applications (3 s) at 0·1 Hz induced a rapid and almost complete suppression of the Ca^{2+} transients, suggesting considerable depletion of the Ca^{2+} stores. A test caffeine pulse applied 44 s after the last caffeine application evoked a small response, indicating that the stores have not yet refilled appreciably (*A*). However, when the test pulse was preceded by a KCl pulse (80 mM in the pipette, open arrowhead), which caused a marked rise in $[Ca^{2+}]_i$, the evoked Ca^{2+} transient recovered completely (*B*). This indicates that Ca^{2+} entry through voltage-gated Ca^{2+} channels accelerates the refilling of the Ca^{2+} stores.

intact pyramidal cells loaded with fura-2 AM. Immediately after applying a series of caffeine pulses, which led to run-down of the Ca^{2+} transient, KCl was pressure applied from a nearby pipette (containing 80 mM KCl). In all cases, the KCl-induced rise in $[Ca^{2+}]_i$ (presumably due to depolarization-induced Ca^{2+} entry through voltage-activated channels), was followed by a prompt recovery of the response to caffeine (Fig. 6*B*). A similar effect was seen when $[Ca^{2+}]_i$ was elevated by a depolarizing voltage pulse in whole-cell clamped neurones (see below Fig. 14; $n = 5$ cells).

These data suggest that caffeine applications empty, rather than desensitize, the ryanodine-sensitive Ca^{2+} stores and that store refilling is accelerated by increasing the availability of cytosolic Ca^{2+}.

CPA and thapsigargin block refilling of ryanodine-sensitive Ca^{2+} stores

Uptake of cytosolic Ca^{2+} into endoplasmic reticulum stores is mediated by Ca^{2+}-ATPases (SERCAs; see Tsien & Tsien, 1990; Pozzan, Rizzuto, Volpe & Meldolesi, 1994), known to be blocked reversibly by cyclopiazonic acid (CPA; Seidler, Jona, Vegh & Martonosi, 1989; Markram *et al.* 1995) and irreversibly by thapsigargin (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990; Pozzan *et al.* 1994). To test the role of this uptake mechanism in the filling of neuronal ryanodine-sensitive Ca^{2+} stores, we examined the effects of these drugs on the spontaneous recovery of the caffeine-induced Ca^{2+} transients following repetitive caffeine applications that caused store depletion. As shown in Fig. 7, recovery in control saline was complete several minutes after the last caffeine pulse. However, when this protocol was repeated during exposure to 20 or 30 μM CPA (Fig. 7*A*; $n = 6$ cells) or to 1 or 3 μM thapsigargin (Fig. 7*B*; $n = 12$ cells), the Ca^{2+} transients failed to recover in all cells tested. Recovery occurred after wash of CPA (Fig. 7*A*). These findings demonstrate that refilling of ryanodine-sensitive Ca^{2+} stores is mediated by SERCAs.

Interestingly, even without prior depletion of the Ca^{2+} stores by caffeine, exposure of the pyramidal cells to CPA for > 7 min completely abolished the caffeine-induced Ca^{2+} transients (Fig. 8; $n = 6$ out of 6 cells). The transients recovered after wash of CPA. These data suggest that the

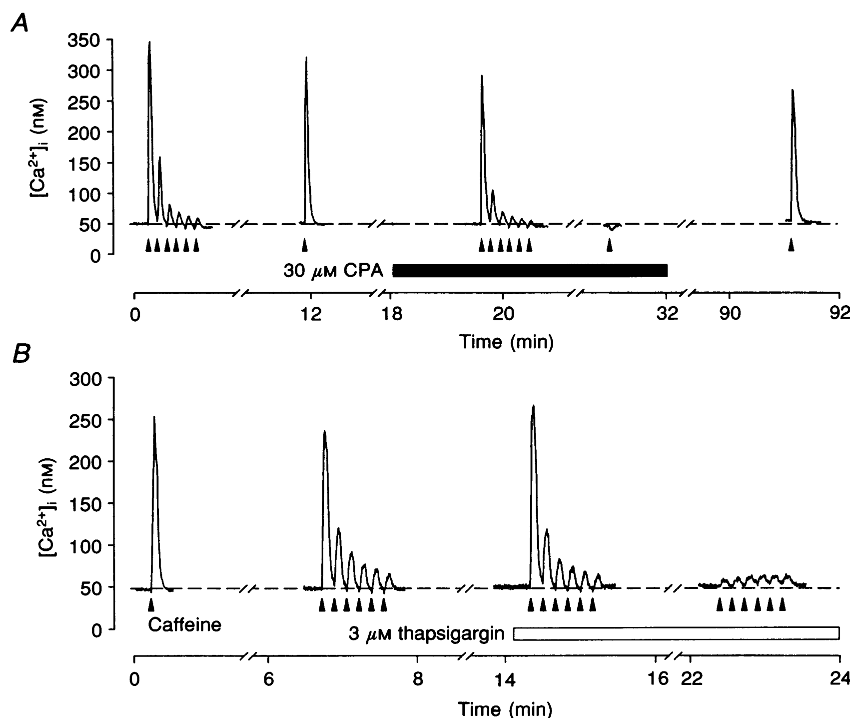

Figure 7. Blockers of endoplasmic reticulum Ca^{2+}-ATPases suppress the refilling of depleted ryanodine-sensitive Ca^{2+} stores

Recordings of somatic $[Ca^{2+}]_i$ in two intact pyramidal cells loaded with fura-2 AM. *A*, six consecutive caffeine applications (3 s, arrowheads 1–6) induced a rapid and almost complete suppression of the Ca^{2+} transients, which recovered spontaneously within 8 min (arrowhead 7). The same protocol was repeated (arrowheads 8–13) a few minutes after 30 μM CPA (bar) was added to the saline. In this condition the Ca^{2+} transients failed to recover (arrowhead 14). Almost complete recovery (73 % of control) was seen only after CPA was washed out for 1 h (arrowhead 15). *B*, in a similar experiment the caffeine-induced Ca^{2+} transients failed to recover after 3 μM thapsigargin (bar) was added to the saline. The effect of thapsigargin was irreversible even with prolonged wash (not shown).

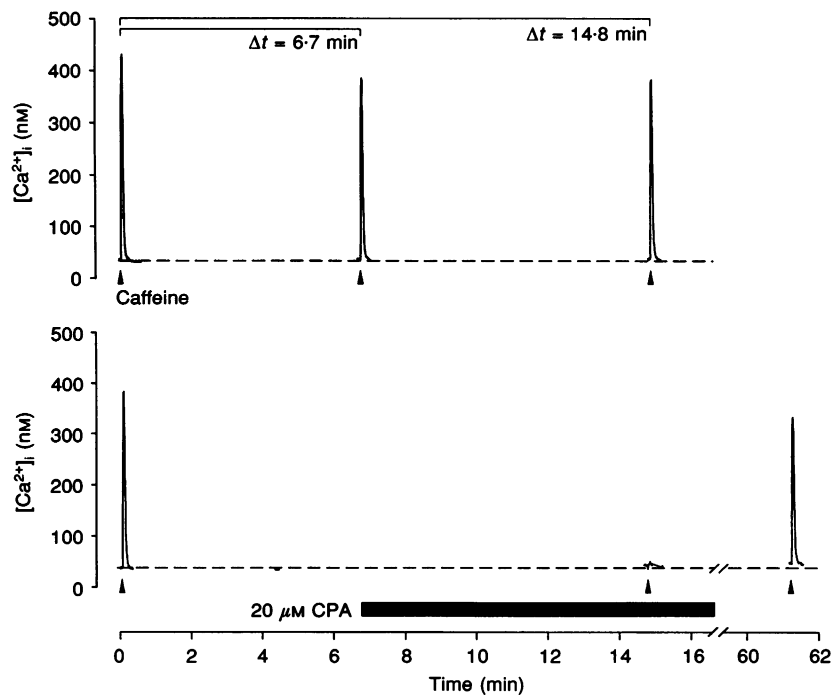

Figure 8. Ryanodine-sensitive Ca^{2+} stores continuously leak Ca^{2+}

Recordings of somatic $[Ca^{2+}]_i$ in an intact pyramidal cell loaded with fura-2 AM. Top panel, two pairs of caffeine pulses (3 s) were applied at 6·7 and at 14·8 min intervals. The Ca^{2+} transients evoked by the conditioning pulses are superimposed. The Ca^{2+} transients evoked by the test pulses are virtually identical, indicating that the lengthy interstimulus intervals allow for almost complete replenishment of the Ca^{2+} stores. Bottom panel, 20 μM CPA (bar) was bath applied after the slice was kept in standard saline for 6·7 min following the last caffeine pulse to ensure refilling of the Ca^{2+} stores. Test caffeine application 8 min later failed to evoke any Ca^{2+} transient, suggesting that the Ca^{2+} stores depleted spontaneously. The caffeine-induced Ca^{2+} transient recovered only after a prolonged washout of CPA.

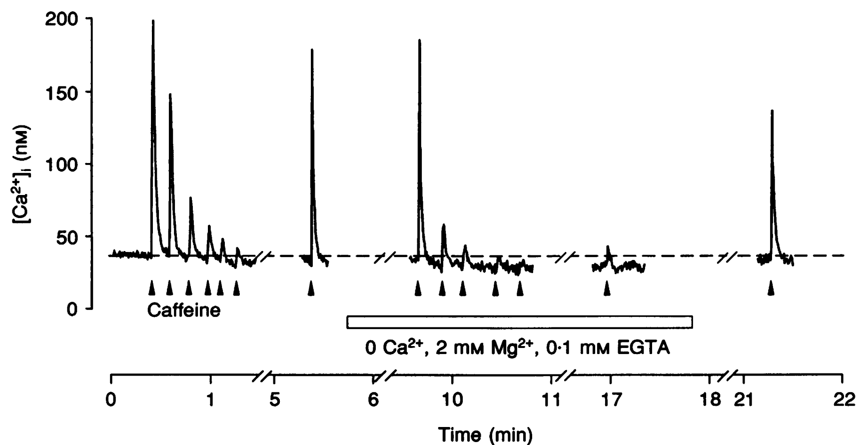

Figure 9. Spontaneous refilling of ryanodine-sensitive Ca^{2+} stores requires extracellular Ca^{2+} ions

Recordings of somatic $[Ca^{2+}]_i$ in an intact pyramidal cell loaded with fura-2 AM. Six consecutive caffeine applications (2 s, arrowheads 1–6) induced a rapid and almost complete suppression of the Ca^{2+} transients, which recovered spontaneously within 5 min (arrowhead 7). The same protocol was repeated (arrowheads 8–12) a few minutes after changing to Ca^{2+}-free saline (shaded horizontal bar), which reduced basal $[Ca^{2+}]_i$ by ~8 nM. In this condition the Ca^{2+} transients decreased much faster and failed to recover spontaneously (arrowhead 14), suggesting that entry of extracellular Ca^{2+} is required for store replenishment. Indeed, after returning to standard saline the caffeine-induced Ca^{2+} signal reappeared (arrowhead 15). Basal $[Ca^{2+}]_i$ also recovered to control level.

ryanodine-sensitive Ca^{2+} stores continuously leak Ca^{2+} and that the loss of Ca^{2+} is counterbalanced by continuous active uptake of Ca^{2+} into the stores.

Refilling of ryanodine-sensitive Ca^{2+} stores depends on extracellular Ca^{2+} influx

In order to characterize further the process of Ca^{2+} uptake into ryanodine-sensitive Ca^{2+} stores, we examined whether extracellular Ca^{2+} is required for spontaneous refilling of the stores. Perfusing the slices with Ca^{2+}-free saline caused a small reduction (15·1 ± 11·3 nM; $n = 6$) in basal $[Ca^{2+}]_i$. As shown in Fig. 9, the decrease in the magnitude of the Ca^{2+} transients induced by repetitive caffeine applications was markedly accelerated in Ca^{2+}-free saline (containing 0·1 mM EGTA), suggesting a more rapid depletion of the ryanodine-sensitive Ca^{2+} stores. Furthermore, recovery of the Ca^{2+} transient was mostly blocked in this condition (6 out of 6 cells), suggesting that the stores do not refill when extracellular Ca^{2+} is removed. Indeed, reperfusion with standard saline prompted the recovery of the Ca^{2+} transient.

To test further the contribution of extracellular Ca^{2+} to the repletion of ryanodine-sensitive Ca^{2+} stores, we examined separately the effects of two broad-spectrum Ca^{2+} channel antagonists, namely Ni^{2+} and D600, on this process (Fig. 10). Adding either 2 mM Ni^{2+} (Fig. 10*A*) or 0·5 mM D600 (not shown) to the saline completely abolished KCl-induced $[Ca^{2+}]_i$ increases, presumably by blocking voltage-activated Ca^{2+} channels in the plasmalemma. This effect was associated with a very small decrease in basal $[Ca^{2+}]_i$

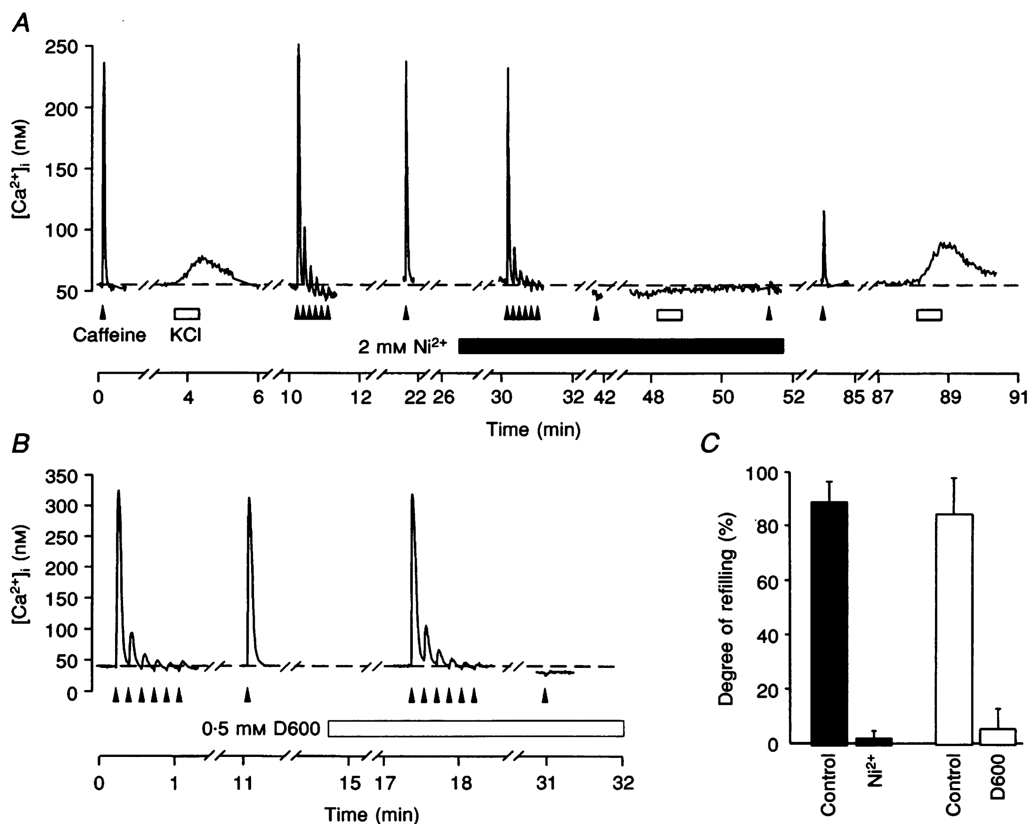

Figure 10. Spontaneous refilling of ryanodine-sensitive Ca^{2+} stores is blocked by blockers of voltage-gated Ca^{2+} channels

A, recordings of somatic $[Ca^{2+}]_i$ in an intact pyramidal cell loaded with fura-2 AM. Bath applications of KCl are marked with open bars. At the beginning of the experiment, six consecutive caffeine applications (3 s, arrowheads 2–7) induced a rapid and almost complete suppression of the caffeine-induced Ca^{2+} transients, which recovered spontaneously within 5 min (arrowhead 8). The same protocol was repeated (arrowheads 9–14) a few minutes after changing to 2 mM Ni^{2+}-containing saline (filled horizontal bar), but in this condition the Ca^{2+} transient failed to recover (arrowheads 15 and 16). The Ni^{2+}-containing saline blocked voltage-dependent Ca^{2+} channels, as indicated by suppression of the KCl-induced increases in $[Ca^{2+}]_i$. Both the caffeine-induced Ca^{2+} signal (arrowhead 17) and the KCl-induced Ca^{2+} response recovered after switching back to standard saline. *B*, in a similar experiment the caffeine-induced Ca^{2+} transients failed to recover after 0·5 mM D600 (shaded horizontal bar) was added to the saline. *C*, a bar histogram depicting the extent of recovery of the caffeine-induced Ca^{2+} transient (reflecting the degree of refilling of the depleted stores) following repetitive caffeine applications before and after adding Ni^{2+} (left bars) or D600 (right bars) to the saline ($n = 6$ in each case). Refilling of the depleted Ca^{2+} stores was markedly suppressed in both cases.

($3{\cdot}0 \pm 2{\cdot}4$ nM in Ni^{2+}, $n = 4$; $8{\cdot}2 \pm 7{\cdot}7$ nM in D600, $n = 9$). Yet in all experiments the drugs almost completely blocked the recovery of the Ca^{2+} transient following store depletion by repetitive caffeine applications (Fig. 10). In contrast, recovery of the Ca^{2+} transient was not blocked by the selective L-type Ca^{2+} channel blocker nitrendipine at the concentration of 10 μM ($n = 5$ cells, not shown).

Taken together, the data suggest that the repletion of the ryanodine-sensitive Ca^{2+} stores depends on Ca^{2+} influx through Ni^{2+}- and D600-sensitive, dihydropyridine-insensitive Ca^{2+} channels operating at resting potential.

The filling state of the ryanodine-sensitive Ca^{2+} stores depends on basal $[Ca^{2+}]_i$

In cerebellar Purkinje cells the amplitudes of caffeine-induced Ca^{2+} transients strongly depend on the basal $[Ca^{2+}]_i$ (Kano *et al.* 1995). We tested whether ryanodine-sensitive Ca^{2+} stores in CA1 pyramidal neurones are similar in this respect. Caffeine-induced Ca^{2+} transients were induced before and after increasing basal $[Ca^{2+}]_i$ with bath-applied KCl (20 or 40 mM). In different experiments, the KCl-elevated basal $[Ca^{2+}]_i$ ranged from 200 to 640 nM. A representative experiment is illustrated in Fig. 11*A*. The initially low level of basal $[Ca^{2+}]_i$ (42 nM) was associated with a caffeine-induced Ca^{2+} transient of 196 nM. After basal $[Ca^{2+}]_i$ was elevated to 284 nM by 40 mM KCl, the Ca^{2+} transient increased to 683 nM (i.e. 348% of control). The decay of the large Ca^{2+} transient was followed by a noticeable undershoot in basal $[Ca^{2+}]_i$ (Fig. 11*A*).

Similar results were obtained in twenty-six pyramidal neurones. These are summarized in the histogram in Fig. 11*B*, showing clearly that the amplitude of caffeine-induced Ca^{2+} transients increases with basal $[Ca^{2+}]_i$. One possible explanation for this relation is that elevated $[Ca^{2+}]_i$ sensitizes the RyRs, so that a greater fraction of stored Ca^{2+} is released by a given caffeine pulse (Bezprozvanny *et al.* 1991; Kano *et al.* 1995). Alternatively, raising $[Ca^{2+}]_i$ may enhance Ca^{2+} uptake into the stores so that more Ca^{2+} is available for release by a caffeine stimulus. To distinguish between these two explanations, the caffeine pulse was applied after the KCl-elevated $[Ca^{2+}]_i$ returned to its initial level. As shown in Fig. 12*A* (upper panel), the evoked Ca^{2+} transient was still supranormal compared with its control. Indeed, recovery of the augmented Ca^{2+} transient to baseline value (Fig. 12*A*, lower panel) lagged 6–9 min behind the peak of the KCl-induced increase in $[Ca^{2+}]_i$ (Fig. 12*B*).

Furthermore, when the experimental protocols described in Figs 11 and 12 were applied sequentially to the very same cell, we observed a marked similarity between caffeine-mediated Ca^{2+} transients evoked immediately after similar KCl applications and transients evoked in conjunction with KCl applications (Fig. 13). The ratio of the corresponding amplitudes of the caffeine-mediated Ca^{2+} transients evoked at high basal levels of $[Ca^{2+}]_i$ and the amplitudes of the Ca^{2+} transients evoked just after the KCl applications was $1{\cdot}14 \pm 0{\cdot}46$ ($n = 7$).

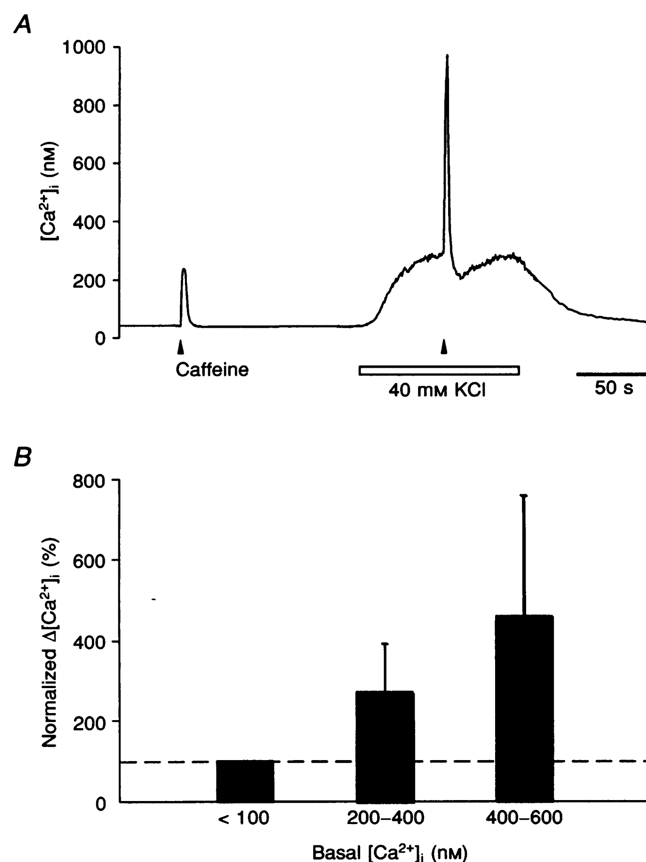

Figure 11. Amplitudes of caffeine-induced Ca^{2+} transients increase with an increase in $[Ca^{2+}]_i$

A, recording of somatic $[Ca^{2+}]_i$ in an intact pyramidal cell loaded with fura-2 AM. Caffeine was applied at rest (arrowhead 1; basal $[Ca^{2+}]_i$ 42 nM) and at the peak of an $[Ca^{2+}]_i$ elevation (284 nM) induced by raising the concentration of KCl in the saline to 40 mM (arrowhead 2). The latter caffeine pulse evoked a 3·5-fold larger Ca^{2+} transient. Note the $[Ca^{2+}]_i$ undershoot following the larger caffeine-induced Ca^{2+} transient. *B*, bar histogram depicting the relation between the size of the caffeine-induced Ca^{2+} transient and the basal $[Ca^{2+}]_i$. The data were obtained from twenty-six experiments identical to the one shown in *A*. The Ca^{2+} transients induced by caffeine while basal $[Ca^{2+}]_i$ was elevated were normalized with respect to the control response evoked before KCl application. Data were segregated into three groups according to the basal $[Ca^{2+}]_i$ at the time caffeine was applied.

These results suggest that ryanodine-sensitive Ca^{2+} stores in CA1 pyramidal cells have a high storage capacity and that the amount of Ca^{2+} sequestered by the stores is directly proportional to the availability of cytosolic Ca^{2+} ions. Thus, transient or sustained elevations of $[Ca^{2+}]_i$ seem to lead to an increased amount of calcium ions stored inside the store.

Depolarization-induced Ca^{2+} transients are not dependent on the filling state of the ryanodine-sensitive Ca^{2+} stores

In some neurones Ca^{2+} entry through voltage-gated Ca^{2+} channels was shown to induce release of Ca^{2+} from ryanodine-sensitive Ca^{2+} stores (Friel & Tsien, 1992; Hua *et al.* 1993; Llano *et al.* 1994). To test for the presence of CICR, we examined whether depolarization-evoked Ca^{2+} transients are affected by depletion of ryanodine-sensitive Ca^{2+} stores (Fig. 14). If CICR contributes to the depolarization-induced Ca^{2+} transients, then these transients should be smaller after store depletion.

In five patch-clamped, fura-2-loaded pyramidal cells, Ca^{2+} transients were evoked by 800 ms depolarizing pulses. A representative experiment is illustrated in Fig. 14*A*. It can be seen that the transients evoked before and after store depletion were nearly the same. One may be concerned, however, by the relatively slow sampling rate (1 Hz) or relatively high intracellular fura-2 concentrations (up to 200 μM) used in these experiments. Therefore, we repeated these experiments in cells loaded with fura-2 AM using pressure KCl applications (80 mM KCl in the application pipette) and 4 Hz sampling rate. (Fig. 14*B*). Also in this case, in which we estimated the intracellular fura-2 concentration to be around 50 μM, the depletion of the caffeine-sensitive stores by six repetitive caffeine applications had virtually no effect on the amplitude of depolarization-induced Ca^{2+} transients. The mean amplitude of the KCl-mediated Ca^{2+} transient after the depletion of the stores related to control was $1{\cdot}01 \pm 0{\cdot}16$ (57 experiments in 36 cells). However, when the data were grouped with respect to the amplitude

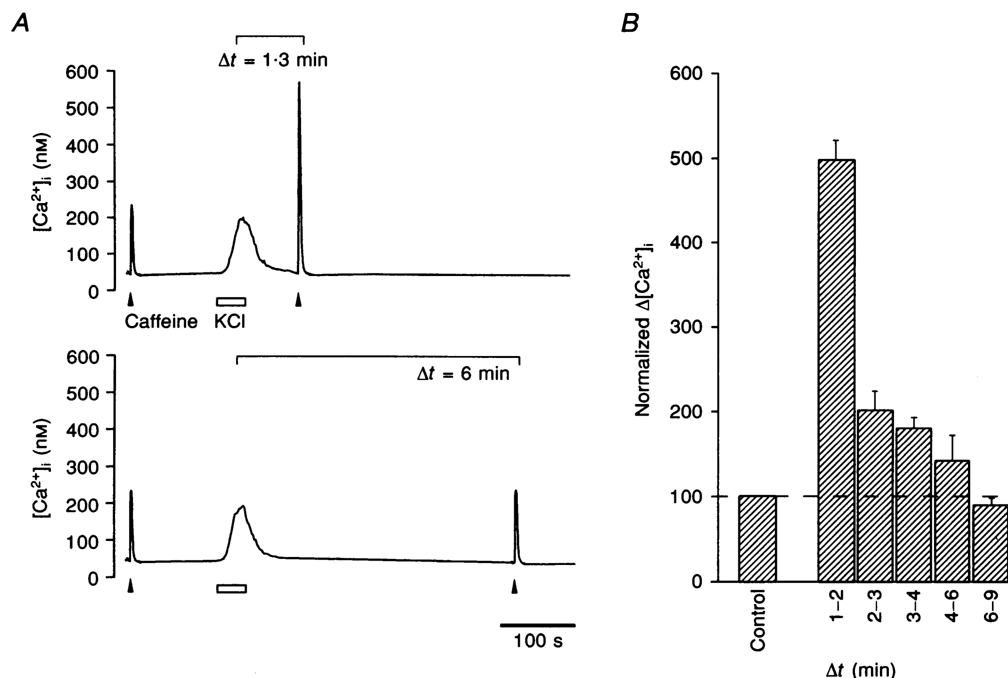

Figure 12. Overcharged ryanodine-sensitive Ca^{2+} stores slowly release excess Ca^{2+} after $[Ca^{2+}]_i$ declines to rest level

A, recording of somatic $[Ca^{2+}]_i$ in an intact pyramidal cell loaded with fura-2 AM. Top panel, caffeine was applied (4 s long application) before and immediately after recovery of the $[Ca^{2+}]_i$ to its baseline value after the peak increase in $[Ca^{2+}]_i$ induced by bath application of 40 mM KCl for 40 s (bar). At the time of the second application basal $[Ca^{2+}]_i$ recovered to its rest level (47 nM), yet the caffeine-induced Ca^{2+} transient was greatly augmented with respect to the control response, suggesting that the Ca^{2+} stores were overcharged. Bottom panel, the same protocol repeated, but the second caffeine pulse was applied 6 min after the KCl-induced $[Ca^{2+}]_i$ peak. The Ca^{2+} transient recovered to control size, suggesting that the Ca^{2+} stores lost surplus Ca^{2+}. *B*, bar histogram depicting the relation between the size of the caffeine-induced Ca^{2+} transient *versus* the time after a KCl-induced $[Ca^{2+}]_i$ elevation (Δt). For convenience, interval Δt was measured between peaks of KCl-mediated and test caffeine-mediated Ca^{2+} transients. Each bar represents a mean of 6–11 data points. Data were derived from thirty-one experiments similar to the one shown in *A*. The amplitudes of the Ca^{2+} transients induced after KCl application were normalized with respect to the control response. The histogram shows that overcharged Ca^{2+} stores slowly release surplus Ca^{2+}.

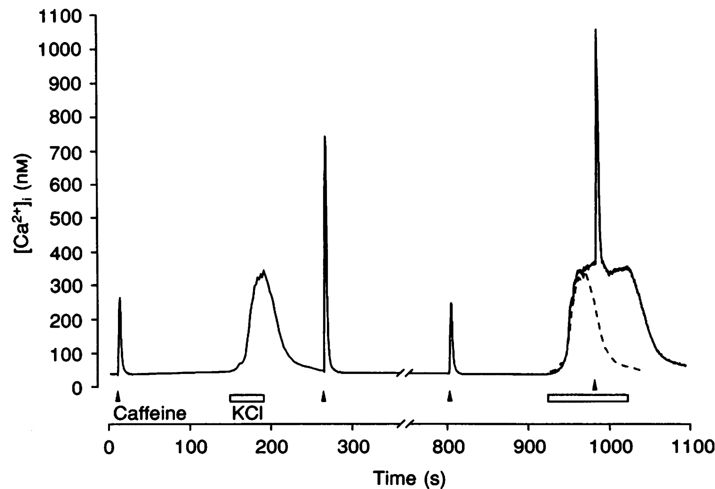

Figure 13. Evidence that the filling state of Ca^{2+} stores determines the magnitude of the caffeine-mediated Ca^{2+} transient

Caffeine was applied (3 s application) before and immediately after recovery of the $[Ca^{2+}]_i$ to its baseline value after the peak increase in $[Ca^{2+}]_i$ induced by bath application of 40 mM KCl for 45 s (bar). After approximately 9 min the experiment was repeated, but this time the second caffeine application was given on top of a 100 s KCl application. For comparison, the waveform of the first KCl-mediated transient increase in $[Ca^{2+}]_i$ was depicted by a dashed line. Note the similar amplitude of the potentiated caffeine-mediated Ca^{2+} transients evoked just after or during KCl-induced elevations of $[Ca^{2+}]_i$.

of KCl-mediated Ca^{2+} transients in control, we found a small effect of store depletion for the amplitudes of more than 200 nM (0·89 ± 0·10, $n = 16$) and no effect for the amplitudes of less than 200 nM (1·05 ± 0·16, $n = 41$). There was a significant difference between these two groups of data ($P < 0·001$, Student's two-tailed t test).

Taken together, these data suggest that CICR from ryanodine-sensitive Ca^{2+} stores does not contribute notably to the small depolarization-induced Ca^{2+} transients (amplitude < 200 nM) but may contribute significantly to the Ca^{2+} transients with amplitudes above 200 nM.

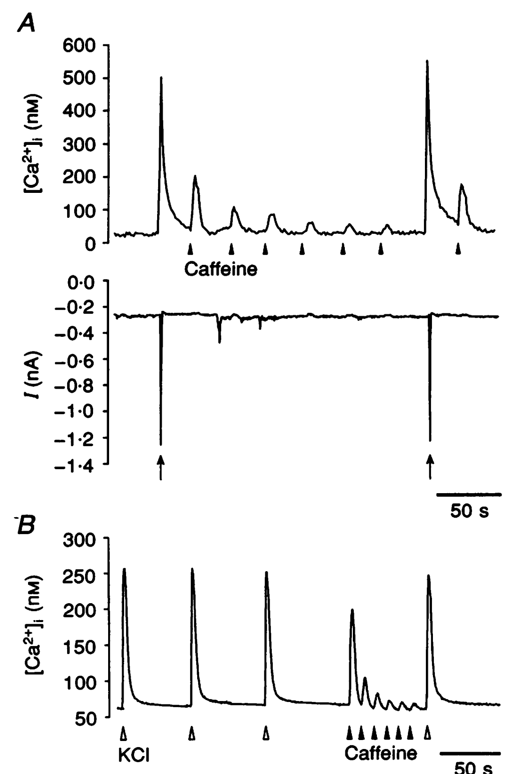

Figure 14. Amplitude of depolarization-induced Ca^{2+} transient is not affected by store depletion

A, simultaneous recordings of the somatic $[Ca^{2+}]_i$ (top trace) and the whole-cell membrane current (bottom trace) in a whole-cell clamped pyramidal cell filled with fura-2. Two depolarizing pulses (from −60 to 0 mV for 800 ms) were applied (arrows), one before and one after six consecutive caffeine applications (3 s) that caused a large decrease in the amplitude of caffeine-induced Ca^{2+} transients. *B*, similar experimental design as in *A*, but the experiment was done in a cell loaded with fura-2 AM. Brief KCl applications (open arrowheads; 3 s, 80 mM KCl in the application pipette) were repetitively delivered in control to ascertain the stability of the amplitude of the induced Ca^{2+} transients. The test KCl application was delivered immediately after the depletion of the ryanodine-sensitive Ca^{2+} stores by six consecutive caffeine applications (each 3 s).

Caffeine evokes local Ca^{2+} transients in the dendrites of pyramidal cells

To examine the distribution of ryanodine-sensitive Ca^{2+} stores in CA1 pyramidal cells, caffeine was locally applied to different parts of the apical dendrites as well as to the soma. An exemplary experiment is illustrated in Fig. 15*A*. Caffeine applied either to the soma (left panel) or to the apical dendrite (right panel) induced a Ca^{2+} transient spatially restricted to the region under the application pipette. The signals evoked in the dendrites (Fig. 15*B*) had amplitudes that were similar to those seen in the soma.

Similar results were obtained at nine application sites in six out of six cells tested. Thus ryanodine-sensitive Ca^{2+} stores also preside in the dendrites of CA1 pyramidal cells and can generate independent, spatially delimited Ca^{2+} transients in response to local caffeine application.

DISCUSSION

The purpose of this study was to gain insight into the functional properties of ryanodine-sensitive Ca^{2+} stores in CA1 hippocampal pyramidal neurones. Using the RyR agonist caffeine as a pharmacological tool, we found that functional Ca^{2+} stores reside in the somata and dendrites of CA1 pyramidal cells. They have a high capacity for sequestering Ca^{2+} and are partially filled at rest. Filling of the stores is mediated by SERCAs and it is controlled by levels of cytosolic Ca^{2+}. Depleted by agonist application, ryanodine-sensitive Ca^{2+} stores refill spontaneously utilizing a Ca^{2+} entry pathway operating at resting membrane potential.

Ryanodine-sensitive Ca^{2+} stores in hippocampal pyramidal cells

Several lines of evidence support the notion that caffeine triggered the release of Ca^{2+} from intracellular ryanodine-sensitive Ca^{2+} stores. Firstly, the caffeine-induced Ca^{2+} transients were not associated with an inward transmembrane current. Secondly, these transients persisted in Ca^{2+}-free saline, while voltage-activated Ca^{2+} currents disappeared. Thirdly, caffeine also evoked Ca^{2+} transients in cells which were held at +60 mV (approximate Ca^{2+} reversal potential), at which transmembrane Ca^{2+} influx is supposedly negligible. Finally, the action of caffeine was completely blocked by 10 or 20 μM ryanodine. We have previously shown (Garaschuk *et al.* 1996) that intracellular dialysis with 20 μM Ruthenium Red, known to block Ca^{2+}

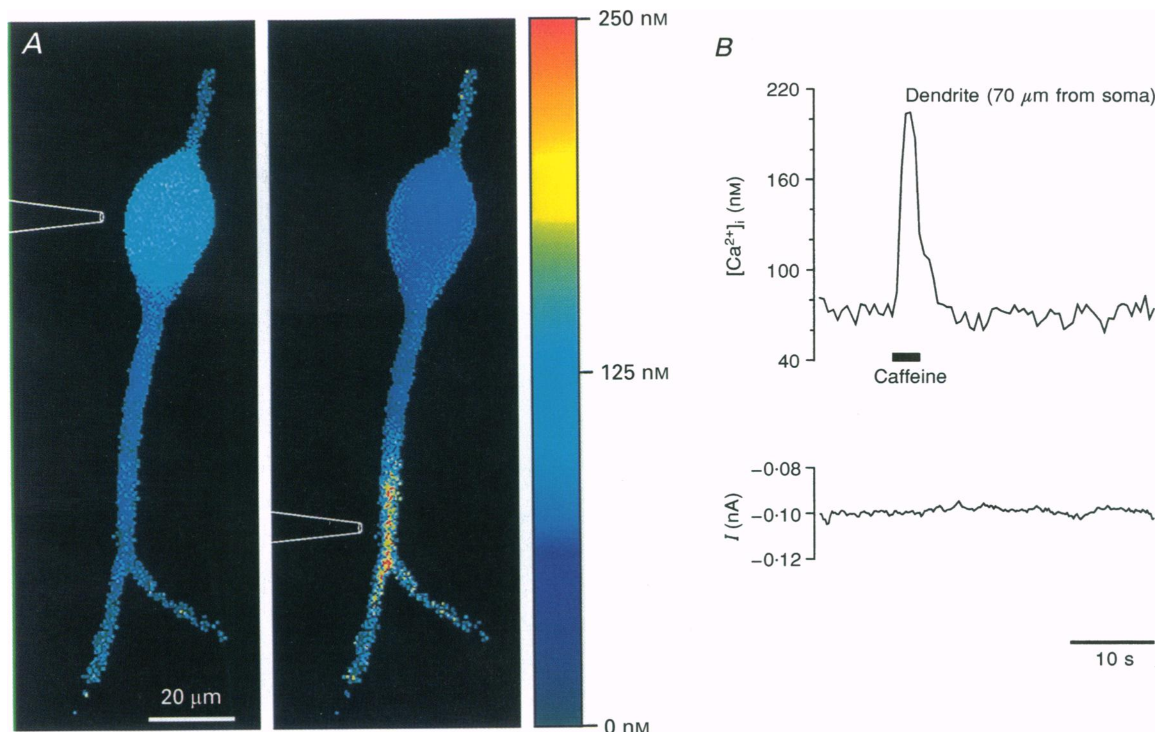

Figure 15. Caffeine causes localized Ca^{2+} changes in somata and dendrites of CA1 pyramidal neurones

A, pseudocolour fluorescence images of $[Ca^{2+}]_i$ illustrating caffeine-induced Ca^{2+} transients in soma (left panel) and apical dendrite (right panel) of a whole-cell clamped CA1 pyramidal cell filled with 150 μM fura-2. The positions of the application pipettes are indicated. Caffeine was applied for 2 s to the soma and for 800 ms to the dendrite. *B*, simultaneous recordings of the dendritic $[Ca^{2+}]_i$ (top trace) and the whole-cell membrane current (bottom trace) in another whole-cell patch-clamped CA1 neurone. Caffeine was applied (3 s application, bar) to the dendrite about 70 μm from the soma.

release from ryanodine-sensitive stores (Henzi & MacDermott, 1992; Ehrlich, Kaftan, Bezprozvannaya & Bezprozvanny, 1994), also blocked the Ca^{2+} responses to caffeine in CA1 pyramidal neurones.

It should be stressed, that in the present study caffeine evoked large Ca^{2+} responses even without prior 'loading' of intracellular Ca^{2+} stores via activation of voltage-activated Ca^{2+} channels. Likewise, in a recent study in cultured mouse hippocampal neurones (Seymour-Laurent & Barish, 1995), pressure-applied caffeine unconditionally evoked conspicuous Ca^{2+} transients. Thus, the ryanodine-sensitive Ca^{2+} stores in hippocampal pyramidal cells contain a releasable pool of Ca^{2+} even at rest. This conclusion is incongruent with a previous study in cultured hippocampal neurones (Shmigol *et al.* 1994), which suggested that ryanodine-sensitive Ca^{2+} stores are empty at rest. The apparent discrepancy may arise from the slow bath-application technique used in the latter study. Indeed, we also found that inclusion of 20 mM caffeine in the perfusing saline evoked much smaller Ca^{2+} responses (O. Garaschuk, Y. Yaari & A. Konnerth, unpublished observations). These data suggest that the rate of Ca^{2+} clearance from the cytosol may be nearly as fast as the rate of Ca^{2+} release by bath-applied caffeine. Therefore, rapid caffeine applications, as used in this study, are more appropriate to resolve functional states of ryanodine-sensitive Ca^{2+} stores.

When pyramidal neurones were dialysed with K^+-containing intracellular solution designed to spare K^+ channel permeability, caffeine-induced Ca^{2+} transients concurred with an outward current. This current, not seen in neurones dialysed with Cs^+, TEA-containing saline, most probably represents a K^+ current activated by the elevated $[Ca^{2+}]_i$. A caffeine-induced Ca^{2+}-activated K^+ current was shown earlier in peripheral and central neurones including hippocampal pyramidal cells (Sah & McLachlan, 1991; Uneyama, Munakata & Akaike, 1993). The absence of a noticeable delay between the rise in $[Ca^{2+}]_i$ and the activation of the outward current suggests that Ca^{2+} is released near the plasmalemma. Indeed, neuronal endoplasmic reticulum may extend very close to this membrane (Henkart, Landis & Reese, 1976).

Release and sequestration of Ca^{2+} by ryanodine-sensitive Ca^{2+} stores

When two identical caffeine pulses were applied in rapid succession, the second caffeine-evoked Ca^{2+} transient was smaller than the first. Likewise, a short series of caffeine pulses resulted in a progressive run-down of the evoked Ca^{2+} responses. Subsequent recovery of these responses occurred spontaneously over 3–4 min (with a time constant of 59 s, assuming mono-exponential recovery process), but could be accelerated by interposing a depolarizing voltage or KCl pulse. Thus the use-dependent decrease of the Ca^{2+} transients

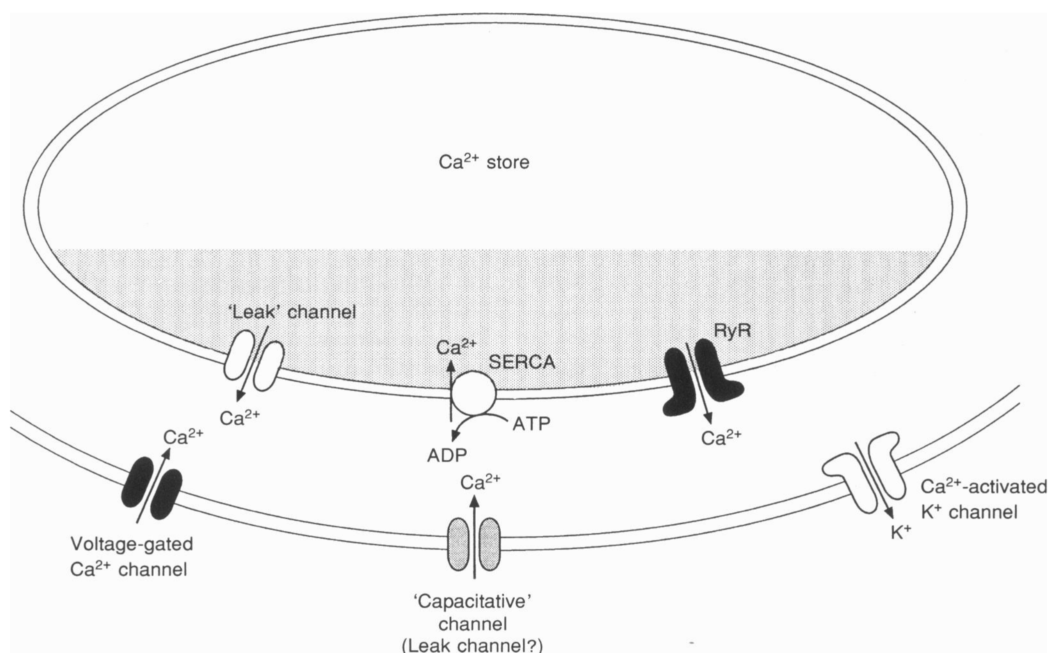

Figure 16. Scheme summarizing the properties of ryanodine-sensitive Ca^{2+} stores in hippocampal pyramidal cells

The stores are partially filled at rest and have high additional capacity for sequestering Ca^{2+}. The main routes for Ca^{2+} transport across the endoplasmic reticulum membrane and the plasmalemma which contribute to the sequestration and the release of Ca^{2+} by these stores are indicated. See text for further explanation.

most probably reflects depletion of the ryanodine-sensitive Ca^{2+} stores, rather than desensitization of the caffeine-binding RyRs.

Exposure of the pyramidal cells to the SERCA blockers thapsigargin and CPA abolished refilling of the depleted Ca^{2+} stores. This finding suggests that Ca^{2+} sequestration in ryanodine-sensitive Ca^{2+} stores is mediated predominantly by SERCAs. Even without prior store depletion, the responses to caffeine were abolished in CPA-containing saline, suggesting that ryanodine-sensitive Ca^{2+} stores are spontaneously leaking, and that maintenance of these stores in a filled state requires continuous Ca^{2+} pumping by the SERCAs.

Raising basal $[Ca^{2+}]_i$ with high-K^+ saline augmented the size of the caffeine-induced Ca^{2+} transients, as recently shown also in cerebellar Purkinje neurones (Kano *et al.* 1995). One explanation for this finding is that cytosolic Ca^{2+} interacts co-operatively with caffeine at the ryanodine receptor to augment the open probability of the receptor channel (Bezprozvanny *et al.* 1991), thereby increasing the release of stored Ca^{2+}. Alternatively, elevated basal $[Ca^{2+}]_i$ may enhance Ca^{2+} sequestration into the stores, so that more Ca^{2+} is available for release by caffeine. Our data showing that the caffeine-induced Ca^{2+} transients were similarly augmented immediately after basal $[Ca^{2+}]_i$ declined to control value, and remained elevated up to 6 min thereafter (Figs 12 and 13), favours the enhanced Ca^{2+} sequestration hypothesis.

Extracellular Ca^{2+} entry into ryanodine-sensitive Ca^{2+} stores

Perfusing the slices with Ca^{2+}-free saline or with saline solutions containing the Ca^{2+} channel antagonists Ni^{2+} or D600, prevented the spontaneous refilling of depleted ryanodine-sensitive Ca^{2+} stores. This finding seen in both fura-2 AM-loaded and in whole-cell patch-clamped (holding potential, −60 mV) pyramidal neurones, demonstrates that store refilling requires a Ca^{2+} influx pathway that is active at the resting membrane potential.

To account for this Ca^{2+} influx, we hypothesize the existence of a class of Ca^{2+} channels in the plasmalemma, activated by depletion of ryanodine-sensitive Ca^{2+} stores. This mechanism would be analogous to the 'capacitative' Ca^{2+} entry in non-neural cells, activated by agonist-induced depletion of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores (see Tsien & Tsien, 1990; Berridge, 1995). Interestingly, Ni^{2+} (2 mM), which blocked refilling of depleted ryanodine-sensitive Ca^{2+} stores, is an effective blocker of capacitative Ca^{2+} entry in non-neural cells (Tsien & Tsien, 1990; Hoth & Penner, 1993).

Capacitative Ca^{2+} entry in some non-neural cells is manifested by a small inward current and an associated rise in $[Ca^{2+}]_i$ (Hoth & Penner, 1993). In CA1 pyramidal cells we did not detect a significant inward current or change in $[Ca^{2+}]_i$ associated with the refilling of depleted ryanodine-sensitive Ca^{2+} stores. Possibly the route of capacitative-like Ca^{2+} entry is spatially restricted to cellular microdomains, where the endoplasmatic reticulum encroaches on the plasmalemma, rendering it invisible to the indicator dye (Putney, 1986; Tsien & Tsien, 1990).

In addition to the capacitative-like Ca^{2+} entry pathway, operating at the resting membrane potential, voltage-gated Ca^{2+} channels represent a powerful mechanism responsible for the activity-dependent refilling of ryanodine-sensitive Ca^{2+} stores. Indeed, ryanodine-sensitive Ca^{2+} stores in hippocampal pyramidal cells refilled promptly following the activation of voltage-gated Ca^{2+} channels. This finding is in agreement with properties of caffeine-sensitive stores found in other types of peripheral and central neurones (Brorson *et al.* 1991; Friel & Tsien, 1992; Shmigol *et al.* 1994).

In conclusion, the refilling of the ryanodine-sensitive Ca^{2+} stores in hippocampal pyramidal cells critically depends upon transmembrane Ca^{2+} entry. Two distinct mechanisms, the newly identified capacitative-like Ca^{2+} entry pathway and voltage-activated Ca^{2+} channels, are utilized for the Ca^{2+} entry at rest and during neuronal activity.

Role of ryanodine-sensitive Ca^{2+} stores in shaping physiological Ca^{2+} transients

The scheme in Fig. 16 represents a hypothetical framework for the results of the present study. It shows that high capacity ryanodine-sensitive Ca^{2+} stores residing in the endoplasmic reticulum communicate bidirectionally with the cytosol via the SERCAs on the one hand and the 'leak' and RyR channels on the other hand.

The SERCAs pump cytosolic Ca^{2+} into the ryanodine-sensitive Ca^{2+} stores, thereby creating a Ca^{2+} concentration gradient across the endoplasmic reticulum membrane. Their activity appears to be regulated by cytosolic $[Ca^{2+}]_i$. At rest, basal $[Ca^{2+}]_i$ is low and the stores are only partially filled (Fig. 16). Elevation in $[Ca^{2+}]_i$, as caused by transmembrane Ca^{2+} entry through voltage-gated Ca^{2+} channels, enhances SERCA activity and increases the amount of stored Ca^{2+}. Through this action the ryanodine-sensitive Ca^{2+} stores may act as Ca^{2+} sinks, participating in the clearance of excess cytosolic Ca^{2+} associated with neuronal activity (e.g. Markram *et al.* 1995).

The ryanodine-sensitive Ca^{2+} stores constantly leak Ca^{2+} back into the cytosol. Therefore, physiological or pharmacologically induced decreases in SERCA activity lead to slow dissipation of the Ca^{2+} gradient across the endoplasmic reticulum membrane and to store depletion. The leak of Ca^{2+} does not show up as an increase in basal $[Ca^{2+}]_i$, indicating that it is effectively removed out of the cell or buffered within the cell by other mechanisms (not illustrated in Fig. 16). In contrast, stimulation of RyR channels leads to a rapid discharge of stored Ca^{2+}, which appears as a large transient increase in $[Ca^{2+}]_i$. This Ca^{2+} transient occurs near the plasmalemma, because it triggers an outward K^+

current via Ca^{2+}-activated K^+ channels (Fig. 16). The size of the Ca^{2+} transient reflects the amount of Ca^{2+} stored prior to RyR stimulation.

Subsequent replenishment of the stores is achieved by the activity of the SERCAs and depends critically on extracellular Ca^{2+} entry through putative 'capacitative' Ca^{2+} channels in the plasmalemma (Fig. 16).

Role of ryanodine-sensitive Ca^{2+} stores in CICR

Contrasting the view that neuronal ryanodine-sensitive Ca^{2+} stores are essentially buffers of cytosolic Ca^{2+}, is the view that these stores participate in the boosting of incoming Ca^{2+} signals via a CICR mechanism (Hua *et al.* 1993; Llano *et al.* 1994; Shmigol, Verkhratsky and Isenberg, 1995; Kano *et al.* 1995), and thus may have a crucial role in neuronal plasticity (Schiegg *et al.* 1995; Reyes & Stanton, 1996; Wang *et al.* 1996) and excitotoxicity (see Mody & MacDonald, 1995). This view contends that, like in cardiac muscle (Nabauer *et al.* 1989), cytosolic Ca^{2+} acts as a RyR agonist to discharge Ca^{2+} from the stores.

In this study, depleting or blocking the ryanodine-sensitive Ca^{2+} stores (with caffeine or ryanodine) did not significantly alter the amplitude and waveform of depolarization-induced Ca^{2+} transients (Figs 3 and 14). This would suggest that CICR does not contribute to these Ca^{2+} transients under our experimental conditions. It should be noted, however, that our experimental conditions were not optimal for evoking the CICR process. In our experiments we evoked rather small depolarization-induced Ca^{2+} transients (up to 500 nM) resulting from relatively small transmembrane Ca^{2+} currents (0·5–1·5 nA). In skeletal muscle, for example, the threshold for CICR was found to be 1·5–2 μM (see Henzi & MacDermott, 1992) and the CICR in cerebellar Purkinje cells (Llano *et al.* 1994) was evoked by the transmembrane Ca^{2+} currents of 4–8 nA giving rise to Ca^{2+} transients with amplitudes reaching 1–2·5 μM. Moreover, no CICR was found in cerebellar Purkinje cells when the amplitude of depolarization-induced Ca^{2+} transient was less that 500 nM (Llano *et al.* 1994; Kano *et al.* 1995).

It can be concluded, therefore, that in central neurones CICR does not contribute significantly to depolarization-induced Ca^{2+} transients evoked by low frequency activity. However, according to a recent study (Alford *et al.* 1993) CICR from ryanodine-sensitive Ca^{2+} stores seems to generate most (65%) of the Ca^{2+} transient in CA1 pyramidal cell dendrites during tetanic activation of excitatory synapses. Thus, CICR may be a major source for the Ca^{2+} transients associated with intense neuronal discharges.

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