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Store depletion-induced calcium influx in rat cerebellar astrocytes

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1 In rat cerebellar astrocytes, intracellular Ca^{2+} store depletion by receptor agonists or sarco(endo)plasmic reticulum Ca^{2+} ATPase inhibitors induced a transient increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the absence of extracellular Ca^{2+} and a sustained increase in its presence.

2 After 10 min treatment with thapsigargin, the $[Ca^{2+}]_i$ was unaffected by removal of thapsigargin, but fell rapidly to the basal level when extracellular Ca^{2+} was removed, suggesting the involvement of capacitative Ca^{2+} entry (CCE); this effect was not seen until cells had been exposed to thapsigargin for at least 2 min.

3 Using the whole cell voltage clamp technique, a 60–100 pA inward current was activated by store depletion, the reversal potential ranging from −5 to 0 mV.

4 When extracellular Na^+ was isotonically replaced by Tris, the thapsigargin-induced $[Ca^{2+}]_i$ increase was enhanced, while the inward current was reduced, indicating that store-operated Ca^{2+} channels were permeable to Na^+; however, they were not permeable to Sr^{2+} or Ba^{2+}.

5 Thapsigargin-induced CCE remained the same in the presence of nifedipine, La^{3+} or Cd^{2+}, while it was inhibited in the presence of SK&F96365.

6 In cerebellar astrocytes, inhibition of protein serine/threonine phosphorylation promoted CCE.

7 In conclusion, in rat cerebellar astrocytes, store depletion activated a CCE *via* channels which were permeable to Ca^{2+} and Na^+ and regulated by phosphorylation.
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Keywords: Capacitative Ca^{2+} entry; intracellular Ca^{2+} concentration; store-operated Ca^{2+} channel; cerebellar astrocytes; thapsigargin; phosphorylation

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CCE, capacitative Ca^{2+} entry; CIF, calcium influx factor; PMA, phorbol 12-myristate 13-acetate; SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase; SOC, store-operated Ca^{2+} channel; VOC, voltage-operated Ca^{2+} channel

Introduction

Increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) play pivotal roles in the regulation of many cellular functions, including neurotransmission, secretion, contraction, motility, adhesion, proliferation, differentiation, gene expression, and cell death, and cells have therefore evolved distinct mechanisms to control rapidly and precisely such changes (Berridge, 1997). A low resting $[Ca^{2+}]_i$ can be maintained either by extrusion of Ca^{2+} across the plasma membrane or by its sequestration into intracellular organelles (Carafoli, 1987). The ATP-dependent Ca^{2+} pump and the Na^+/Ca^{2+} exchanger, localized in the plasma membrane, are responsible for Ca^{2+} extrusion (Gill *et al*., 1984), while the endoplasmic reticulum (ER) is believed to be the major organelle that stores Ca^{2+} *via* a Ca^{2+} pump (Pozzan *et al*., 1994).

Ca^{2+} influx from the extracellular compartment and Ca^{2+} release from intracellular Ca^{2+} stores both contribute to increases in the $[Ca^{2+}]_i$. In excitable cells, voltage-operated Ca^{2+} channels (VOC) are mainly responsible for the influx of extracellular Ca^{2+} seen on membrane depolarization (Hess, 1990). In response to a receptor agonist, two distinct mechanisms, Ca^{2+} release from intracellular Ca^{2+} stores *via* IP_3 generation and Ca^{2+} influx *via* receptor-operated Ca^{2+} channels, are responsible for the increased $[Ca^{2+}]_i$ (Marks, 1997; Furuichi & Mikoshiba, 1995; Barnard, 1996). Thus, the intracellular Ca^{2+} stores have a dual function as a Ca^{2+} buffer, removing Ca^{2+} from the cytosol by an ATP-dependent process and releasing Ca^{2+} in response to receptor activation, thus causing an increase in the $[Ca^{2+}]_i$. On receptor activation, the Ca^{2+} permeability of the plasma membrane increases after depletion of the intracellular Ca^{2+} stores (Neher, 1992; Clapham, 1993); this Ca^{2+} influx pathway linked to a reduced store Ca^{2+} content is termed capacitative Ca^{2+} entry (CCE) and occurs *via* so-called store-operated Ca^{2+} channels (SOC) (Putney, 1990).

CCE has been identified in many cells (for review, see Parekh & Penner, 1997; Putney, 1999; Barritt, 1999), but the mechanism linking store-depletion and the increased Ca^{2+} permeability of the plasma membrane remains elusive. Several mechanisms have been proposed. In mast cells, an inward Ca^{2+} current (I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current) is seen following depletion of intracellular Ca^{2+} stores (Hoth & Penner, 1992), while, in mouse pancreatic acinar cells, CCE results from a I_{CRANC}, Ca^{2+} release-activated nonselective cation current (Krause *et al*., 1996). In *Drosophila*, the trp genes may encode SOCs (Zhu *et al*., 1996; Harteneck *et al*., 2000). It has been suggested that a Ca^{2+} influx factor (CIF), generated when stores are depleted,

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diffuses to the plasma membrane where the SOC is located, and activates CCE (Randriamampita & Tsien, 1993). Alternatively, direct physical contact between the plasma membrane and the endoplasmic reticulum IP_3 receptor, which detects depletion of the intracellular Ca^{2+} stores, might be responsible for activating CCE (Irvine, 1990). Recently, two studies have provided evidence to support this latter hypothesis of an exocytosis-like mechanism of CCE activation (Yao *et al*., 1999; Patterson *et al*., 1999).

In this study, using primary cultures of rat cerebellar astrocytes, we characterized store depletion-induced Ca^{2+} influx and found that CCE is involved in the Ca^{2+} signalling process in these cells and is inhibited by protein serine/threonine phosphorylation.

Methods

Cell culture

Primary cultures of rat cerebellar astrocytes were prepared from 7-day-old Sprague-Dawley rat cerebellum, as described previously (Gallo *et al*., 1982). Briefly, the cells were dissociated in a Ca^{2+}-free buffer containing 0.25 mg ml^{-1} of trypsin and 2400 units ml^{-1} of DNase, and collected by centrifugation before being plated on poly-L-lysine-coated 24 mm glass coverslips (1×10^6 cells/coverslip) and grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. The freshly isolated cells from the cerebellum contained both granule neurons and glial cells. To obtain granule neuron-enriched cultures, the growth medium was supplemented with 21.5 mM KCl and 10 μM cytosine arabinoside to minimize non-neuronal cell proliferation. In the absence of high K^+ and cytosine arabinoside, the number of non-neuronal cells was increased. The non-neuronal cells used in this study were identified as astrocytes by positive immunocytochemical staining for glial fibrillary acidic protein and their distinct morphology (data not shown). Astrocyte-enriched cultures were grown in basal Eagle's medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 units ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin. Experiments were performed on 8–10 day cultures.

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

The $[Ca^{2+}]_i$ change in a single cell was measured using the fluorescent Ca^{2+} indicator, fura-2, as described previously (Grynkiewicz *et al*., 1985; Chin & Chueh, 1998). Briefly, cells grown on glass coverslips were loaded with fura-2 by incubation for 20 min at 37°C with 10 μM fura-2 acetoxymethyl ester and 1% pluronic acid in loading buffer (in mM: NaCl 150, KCl 5, glucose 5, $CaCl_2$ 2.2, $MgCl_2$ 1 and HEPES 10, pH 7.4). The coverslips were then mounted in a modified Cunningham chamber (Cunningham & Szenberg, 1968) attached to the stage of a Nikon diaphot inverted microscope equipped with a Nikon 40× fluor objective, and cell fluorescence monitored using a dual-excitation spectrofluorometer with a photomultiplier-based detection system (Spex Industries, Edison, NJ, U.S.A.). Only a single cell per coverslip, selected by a pinhole diaphragm placed in the image plane in front of the photomultiplier, was used in experiments; this was excited alternately with 340 and 380 nm light, and the emitted fluorescent light collected by the objective through a 510 nm long-wave pass filter. The $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence at 340 nm and 380 nm according to the equation derived by Grykiewicz *et al*. (1985) using parameters obtained on our instrument for fura-2 in rats cerebellar astrocytes: $R_{min} = 0.68$; $R_{max} = 5.42$; $S_{f2}/S_{b2} = 4.62$; $K_d = 135$ nM. In experiments to measure Ba^{2+} or Sr^{2+} influx, the $CaCl_2$ in the loading buffer was replaced with $BaCl_2$ or $SrCl_2$, respectively. Under nominally Ca^{2+}-free conditions, extracellular Ca^{2+} was omitted from the loading buffer after fura-2 loading. All experiments were performed on at least 18 cells. The results of one representative experiment are illustrated graphically and the mean ± s.d. values for the $[Ca^{2+}]_i$ increase, calculated for n cells of different batches, are given in the text.

Examination of fluorescence quenching by Mn^{2+}

Fura-2-loaded cells, grown on coverslips, were transferred to loading buffer containing 0.2 mM Ca^{2+} (low calcium loading buffer; LCL buffer). They were then incubated for 5 min in LCL buffer alone or LCL buffer containing 100 nM staurosporine, 100 nM phorbol 12-myristate 13-acetate (PMA), or 2 nM okadaic acid, then 1 mM Mn^{2+}, either alone or together with 1 μM thapsigargin, was added, and Mn^{2+} influx monitored by the quenching of fura-2 fluorescence at the isosbestic wavelength, 360 nm. All experiments were performed at least six times, using different batches of cells. The results of one representative experiment are illustrated graphically, while the mean ± s.d. for the fluorescence quenching during the initial 20 s after Mn^{2+} addition, calculated from the number (n) of cells, are shown in Table 1 and were analysed by Student's t-test.

Electrophysiological recording

Thapsigargin-induced currents were measured by the whole cell voltage clamp technique using a patch clamp amplifier (Axopatch 1-D, Axon Instruments, Inc., Foster City, CA, U.S.A.) as described previously (Hamill *et al*., 1981). The recording chamber was superfused with bathing buffer consisting of: (in mM) NaCl 137, CsCl 4, $MgCl_2$ 0.5, $CaCl_2$

Table 1 Effect of protein phosphorylation on thapsigargin-induced Mn^{2+} influx

Pretreatment	*Control cells*	*Thapsigargin-treated cells*
	(*Arbitrary unit cps* $\times 10^{-3}$/20 s)	
None	1.3 ± 0.05	1.9 ± 0.11
Staurosporine	1.4 ± 0.12	8.7 ± 0.14*
PMA	1.4 ± 0.08	1.1 ± 0.09
Okadaic acid	1.5 ± 0.07	1.5 ± 0.04

Data were obtained from Figure 11. Briefly, control cells and cells pretreated with 100 nM staurosporine, 100 nM PMA or 2 nM okadaic acid for 5 min, were stimulated with buffer (control cells) or 1 μM thapsigargin (thapsigargin-treated cells), and the Mn^{2+} influxes were monitored by measuring quenching of fura-2 fluorescence. The net decrease in fluorescence intensity during the initial 20 s after thapsigargin addition was expressed in arbitrary units. The results are the mean ± s.d. ($n = 15$), using different batches of cells. *$P < 0.001$ significantly different from control cells without any pretreatment.

5.4, glucose 5, HEPES 10, pH 7.4, while the pipette buffer consisted of (in mM): CsCl 110, MgATP 5, EGTA 20, tetraethylammonium chloride 20 and HEPES 10, pH 7.2. A gigaohm seal was formed with a borosilicate glass patch pipette with a tip diameter of 1–1.5 μm and an input resistance of 2–5 MΩ. Data were sampled at 2 kHz and filtered at 1 kHz (−3dB cutoff frequency) using an eight-pole Bessel-type filter (VBF/8.03, Kemo, Beckenham, Kent, U.K.). The digitised traces were stored on a magneto-optical disk unit (RMO-S550, Sony, Japan) and analysed using pCLAMP software, version 6.03 (Axon Instruments, Inc.). To record thapsigargin-induced currents, the membrane potential was held at −70 mV and step pulsed for 100 ms to −100 mV at 10 s intervals, currents being recorded before and after thapsigargin perfusion and after washout. To obtain the current-voltage relationship, ramp pulses were given at 5 s intervals from the holding potential of −40 to −100 mV, then to 100 mV, then back to the holding potential at a speed of 2 V/s. In some experiments, extracellular Na^+ was isotonically replaced with Tris. All experiments were repeated six times using different batches of cells with similar results. One set of representative data is shown and the mean ± s.d. values for the current change are given in the text.

Materials

Basal Eagle's medium, foetal bovine serum, glutamine, and penicillin-streptomycin were purchased from Life Technologies, Inc. (Grand Island, NY, U.S.A.). Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.). Thapsigargin, okadaic acid, staurosporine, glutamate, and PMA were purchased from Research Biochemicals International (Natick, MA, U.S.A.). ATP, poly-L-lysine, trypsin, DNase, angiotensin II, EGTA, and cytosine arabinoside were purchased from Sigma (St. Louis, MO, U.S.A.). SK&F96365 was purchased from Biomol (Plymouth Meeting, PA, U.S.A.). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

Results

To determine whether cerebellar astrocytes contained SOCs we first depleted the intracellular Ca^{2+} stores *via* IP_3 generation using ATP or angiotensin II in the presence or absence of extracellular Ca^{2+}. As shown in Figure 1 (traces a and c), in the presence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ increased rapidly from a basal level of 50 ± 22 nM (n = 40) to 856 ± 60 nM (n = 21) or 790 ± 35 nM (n = 19) in response to addition of 100 μM ATP or 100 nM angiotensin II, respectively, then declined to a sustained level of 213 ± 21 nM (n = 21) or 224 ± 16 nM (n = 19) 180 s after addition of ATP or angiotensin II, respectively. In the absence of extracellular Ca^{2+}, the ATP- or angiotensin II-induced $[Ca^{2+}]_i$ increase peaked at 682 ± 47 nM (n = 18) or 486 ± 24 nM (n = 21), respectively, 20 or 38% lower than in the presence of Ca^{2+}, and the effect was transient, with the $[Ca^{2+}]_i$ falling back completely to the basal level (Figure 1, traces b and d).

The sustained $[Ca^{2+}]_i$ phase seen in the presence of extracellular Ca^{2+} represented the influx of Ca^{2+}, probably *via* SOCs, since intracellular Ca^{2+} stores are depleted by ATP- or angiotensin II-induced IP_3 generation. When the

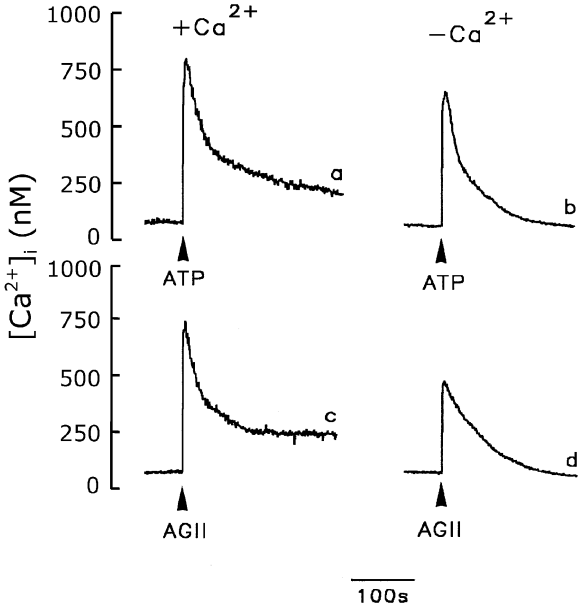

Figure 1 Effect of extracellular Ca^{2+} on the $[Ca^{2+}]_i$ increase in cerebellar astrocytes. The $[Ca^{2+}]_i$ increase induced by 100 μM ATP (traces a and b) or 100 nM angiotensin II (AGII) (traces c and d) were measured in the presence (traces a and c) or absence (traces b and d) of extracellular Ca^{2+}.

intracellular Ca^{2+} stores were depleted using the intracellular Ca^{2+} pump inhibitors, thapsigargin and cyclopiazonic acid, similar results were seen. As shown in Figure 2A, a slow but significant $[Ca^{2+}]_i$ increase was induced by 1 μM thapsigargin (Chin & Chueh, 1998) and reached the peak of 321 ± 40 nM (n = 15) at 210 s in the presence (trace a) or absence (trace b) of extracellular Ca^{2+}. The $[Ca^{2+}]_i$ returned to the basal level in the absence of extracellular Ca^{2+}, while it remained high in its presence. This sustained $[Ca^{2+}]_i$ increase, about 463 ± 41 nM (n = 15) 15 min after thapsigargin addition, represents extracellular Ca^{2+} entry, rather than intracellular Ca^{2+} release, since the $[Ca^{2+}]_i$ was not affected by thapsigargin removal after the cells had been exposed to thapsigargin for 10 min, but immediately declined to the basal level when extracellular Ca^{2+} was removed and returned to the previous sustained level when 2.2 mM Ca^{2+} was added back to the bathing solution (Figure 2B). Identical results were seen using cyclopiazonic acid (10 μM) (Krishna *et al*., 2001) (Figure 3). The basal and sustained $[Ca^{2+}]_i$ was 77 ± 12 nM (n = 15) and 291 ± 35 nM (n = 15), respectively.

Our data indicate that, in rat cerebellar astrocytes, CCE occurs following depletion of the intracellular Ca^{2+} stores. Figure 4 shows the relationship between CCE and the duration of thapsigargin treatment. To detect CCE, we determined whether the $[Ca^{2+}]_i$ decreased after the simultaneous removal of thapsigargin and extracellular Ca^{2+}. As shown in Figure 4 (trace a), when extracellular Ca^{2+} and thapsigargin were removed 1.5 min after exposure of cells to 1 μM thapsigargin, the $[Ca^{2+}]_i$ was unaffected, indicating that Ca^{2+} influx *via* SOCs was not activated and that the $[Ca^{2+}]_i$ increase was attributable to intracellular Ca^{2+} release. However, in another cell after 2 min of thapsigargin treatment, CCE was detected, since the $[Ca^{2+}]_i$ was reduced when thapsigargin and extracellular Ca^{2+} were removed

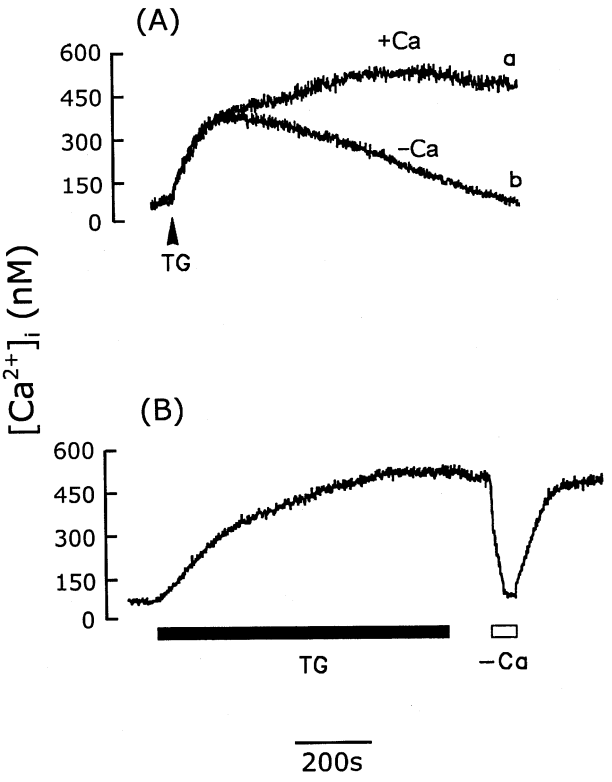

Figure 2 Activation of CCE by thapsigargin in cerebellar astrocytes. (A) $[Ca^{2+}]_i$ increases induced by 1 μM thapsigargin (TG) in the presence (trace a) or absence (trace b) of extracellular Ca^{2+}. (B) After 10 min exposure to thapsigargin (1 μM) (TG), the $[Ca^{2+}]_i$ remained elevated after removal of thapsigargin, but fell to the basal level when extracellular Ca^{2+} was removed and returned to the elevated level when Ca^{2+} was added back to the bathing buffer. Similar results were seen using five different batches of cells ($n=15$).

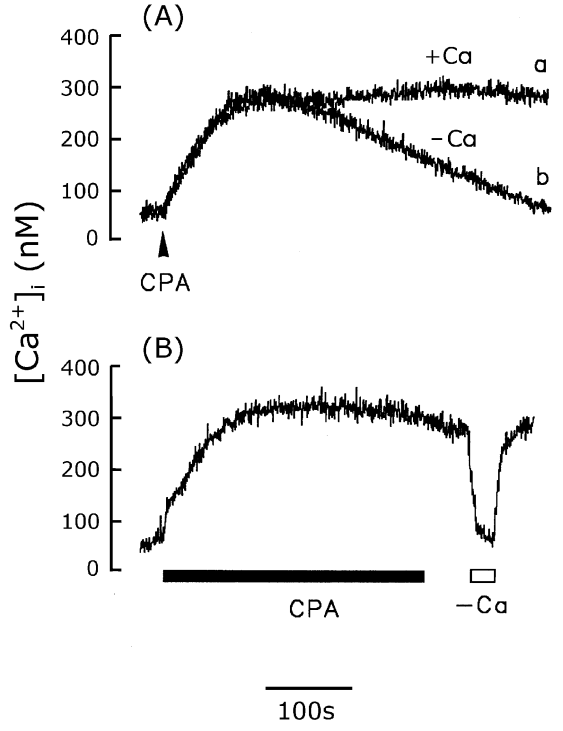

Figure 3 Activation of CCE by cyclopiazonic acid in cerebellar astrocytes. (A) $[Ca^{2+}]_i$ increase induced by 10 μM cyclopiazonic acid (CPA) in the presence (trace a) or absence (trace b) of extracellular Ca^{2+}. (B) After 10 min exposure to CPA, the $[Ca^{2+}]_i$ was unaffected by removal of CPA, but fell to the basal level when extracellular Ca^{2+} was removed. The experiment was repeated five times using different batches of cells with similar results ($n=15$).

(trace b), and this effect became progressively greater the longer the cells were exposed to thapsigargin (traces c–f). In this experiment, each trace used a fresh individual cell to expose thapsigargin for indicated time. These results indicate that longer exposure to thapsigargin resulted in a lower amount of releasable Ca^{2+} within the Ca^{2+} stores and a greater contribution of extracellular Ca^{2+} influx to the thapsigargin-induced increased $[Ca^{2+}]_i$. To confirm that the degree of availability of the releasable Ca^{2+} within the Ca^{2+} pools fell with increased time of exposure to thapsigargin, $[Ca^{2+}]_i$ increase induced by 100 μM ATP was measured 1 min after extracellular Ca^{2+} was removed following exposure of individual cell to thapsigargin for indicated time. As shown in Figure 4 (trace f), virtually no ATP-induced $[Ca^{2+}]_i$ increase was seen after cells were exposed to thapsigargin for 10 min, while a rapid and significant $[Ca^{2+}]_i$ increase was evoked if cells were exposed to thapsigargin for a shorter time period (traces a–c). Figure 5 summarizes the statistical data measured in Figure 4. The dependence of Ca^{2+} influx *via* CCE on thapsigargin exposure time period contrasts with the amplitude of 100 μM ATP-induced $[Ca^{2+}]_i$ increase, an indication of the releasable Ca^{2+} within the Ca^{2+} stores.

In many cells, the SOC is also permeable to Na^+ (Krause *et al*., 1996). We therefore determined the effect of Na^+ on SOC-mediated Ca^{2+} influx in rat cerebellar astrocytes. Figure 6 shows thapsigargin-induced $[Ca^{2+}]_i$ increases measured in Na^+-containing (trace a) and Na^+-free buffer (trace b). As shown in trace b, when extracellular Na^+ was isotonically replaced with Tris and the cells exposed to 1 μM thapsigargin for 5 min, the sustained $[Ca^{2+}]_i$ increase, 310 ± 20 nM ($n=5$), was completely attributable to extracellular Ca^{2+} influx, since the $[Ca^{2+}]_i$ rapidly declined to the basal level, 44 ± 12 nM ($n=5$), when extracellular Ca^{2+} was removed. Subsequent addition of 100 μM ATP did not induce a $[Ca^{2+}]_i$ increase, indicating that the intracellular Ca^{2+} stores were indeed depleted; this contrasts with the situation in Na^+-containing buffer in which 10 min treatment with 1 μM thapsigargin was required to completely deplete the intracellular Ca^{2+} stores (Figure 4, trace f). These results indicate that, under Na^+-free conditions, depletion of the intracellular Ca^{2+} stores was readily achieved, which, in turn, activated CCE.

To further characterize SOC-mediated Ca^{2+} entry and the effect of Na^+ on SOCs, we used the whole cell voltage clamp technique to measure the thapsigargin-induced current by step pulses. The membrane potential was held at -70 mV and step hyperpolarized to -100 mV for 100 ms at 10 s intervals, and the amplitudes of the currents measured at -100 mV before, and after, perfusion with thapsigargin, and after thapsigargin washout were plotted versus time. As shown in Figure 7, following exposure of cells to 1 μM thapsigargin, an average of 80 ± 13 pA ($n=10$) inward current was evoked and was still present after thapsigargin washout (Figure 7B), while no current change was seen after perfusion with buffer alone (Figure 7A). To obtain the current-voltage curve for the thapsigargin-activated current,

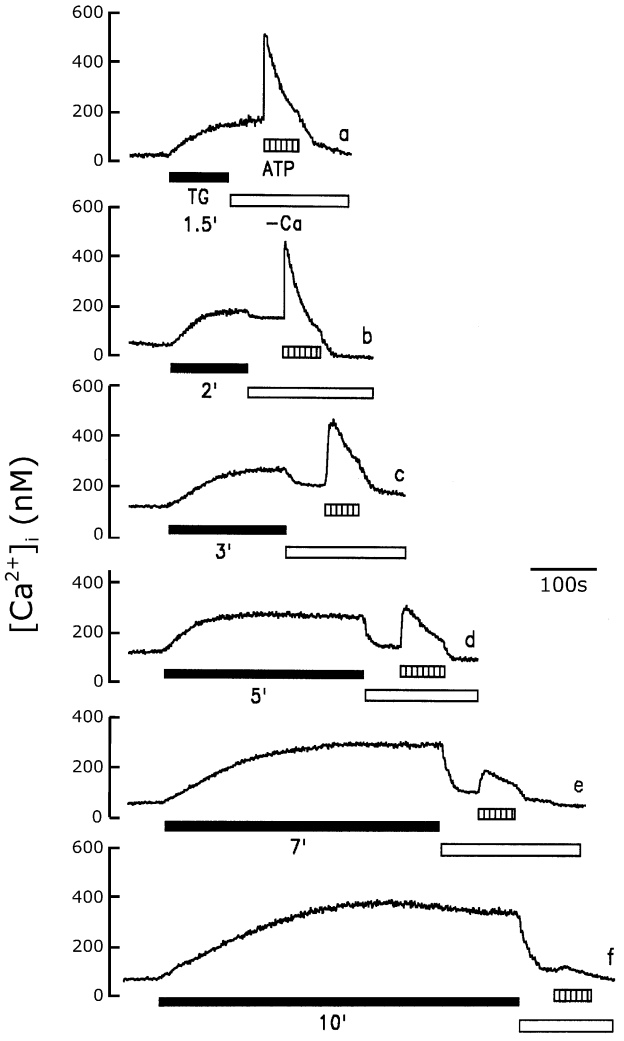

Figure 4 Dependence of CCE on the thapsigargin exposure time. After cells were treated with 1 μM thapsigargin (TG) for 1.5 min (trace a), 2 min (trace b), 3 min (trace c), 5 min (trace d), 7 min (trace e), or 10 min (trace f), as indicated, extracellular Ca^{2+} was removed, then, 1 min later, the ATP (100 μM) was added. $[Ca^{2+}]_i$ increase was measured. The experiment was repeated six times using different batches of cells with similar results.

the current was recorded by voltage ramps spanning from -100 mV to $+100$ mV in 100 ms at 5 s intervals with a holding potential of -40 mV. As shown in Figure 8, the current-voltage curve for the thapsigargin-activated current showed an outward rectification relationship, with the reversal potential ranging from -5 to 0 mV (left panel, middle). The amplitude of the thapsigargin-activated current was reduced when the Na^+ in the bathing solution was isotonically replaced with Tris (right panel, middle), while the reversal potential remained the same. The reduction in current at -100 mV was $24\pm6\%$ ($n=10$) and the reversal potentials were -2.4 ± 0.3 mV ($n=10$) in Na^+ containing and 1.6 ± 0.2 mV ($n=10$) in Tris containing buffer. Similar current-voltage curves were seen after thapsigargin washout in both Na^+- and Tris-containing buffer (bottom panels).

Our data suggested that the SOC in rat cerebellar astrocytes is permeable to both Ca^{2+} and Na^+. We next determined whether it was permeable to Ba^{2+} and Sr^{2+}. If this was the case, Ba^{2+} and Sr^{2+} were not the substrate for Ca^{2+} pump and would accumulate in the cytosol, resulting in a progressive increase in fura-2 fluorescence. As shown in Figure 9 (trace a), in the presence of 2.2 mM $BaCl_2$, the fluorescence change induced by thapsigargin was indistinguishable from that seen in Ca^{2+}-free medium (Figure 2A, trace b). Similar results were obtained when extracellular Ca^{2+} was replaced by Sr^{2+} (Figure 9, trace b).

To further confirm that Ca^{2+} influx following intracellular Ca^{2+} stores depletion we observed in cerebellar astrocytes is due to the activation of SOC rather than VOC, we next determined the sensitivity of this Ca^{2+} influx pathway to nifedipine, Cd^{2+}, and La^{3+}, agents known to block VOC, and SK&F96365, a specific SOC inhibitor. As shown in Figure 10, cerebellar astrocyte was individually treated with buffer, 10 μM nifedipine, 100 μM Cd^{2+}, 100 μM La^{3+}, and 10 μM SK&F96365 for 5 min, 1 μM thapsigargin was then added to each group. Except for cells treated with SK&F96365, a prolonged $[Ca^{2+}]_i$ increase was seen in all cells; the sustained $[Ca^{2+}]_i$ being 306 ± 38 nM ($n=8$), 293 ± 32 nM ($n=8$), 321 ± 41 nM ($n=8$) and 298 ± 34 nM ($n=8$) in cells treated with buffer, nifedipine, Cd^{2+} and La^{3+}, respectively. This prolonged $[Ca^{2+}]_i$ increase was due to Ca^{2+} influx, since it immediately declined to the basal level when extracellular Ca^{2+} was removed and returned to the same sustained level when 2.2 mM Ca^{2+} was added back to the bathing solution. In the presence of SK&F96365, thapsigargin-induced $[Ca^{2+}]_i$ increase was transient, and the $[Ca^{2+}]_i$ returned to the basal level even in the presence of extracellular Ca^{2+}. This is indistinguishable from that seen in Ca^{2+}-free medium (Figure 2A, trace b).

We finally determined whether CCE activity was regulated by protein phosphorylation by examining the effects of staurosporine, a protein kinase inhibitor, PMA, a protein kinase C activator, and okadaic acid, a phosphatase inhibitor, on SOC-mediated Mn^{2+} influx after store depletion. As with Sr^{2+} or Ba^{2+}, once Mn^{2+} enters the cell, it is trapped in the cytosol, and the fluorescence of fura-2 is quenched after it binds to Mn^{2+}. As shown in Figure 11, addition of 1 μM thapsigargin to cerebellar astrocytes caused slight, but significant, fluorescence quenching (trace b) compared to control cells (trace a) and this quenching was slightly reduced following 5 min pretreatment of cells with 1 μM PMA or 2.5 nM okadaic acid (traces f and h, respectively), but significantly potentiated by 1 μM staurosporine (trace d). In control cells, the basal fluorescence quenching was not affected by any of these three drugs (compare trace a with traces c, e, and g). The statistical data are summarized in Table 1.

Discussion

Under physiological conditions, depletion of the intracellular Ca^{2+} stores occurs following receptor occupancy as a result of IP_3 generation, and Ca^{2+} influx *via* the SOC is then activated, resulting in the so-called CCE (Putney, 1990; Berridge, 1997; Parekh & Penner, 1997; Barritt, 1999). In the present study, in rat cerebellar astrocytes, a sustained $[Ca^{2+}]_i$ increase was seen in response to ATP or angiotensin II stimulation in the presence of extracellular Ca^{2+}, but not in its absence (Figure 1). A slow $[Ca^{2+}]_i$ increase was seen following inhibition of

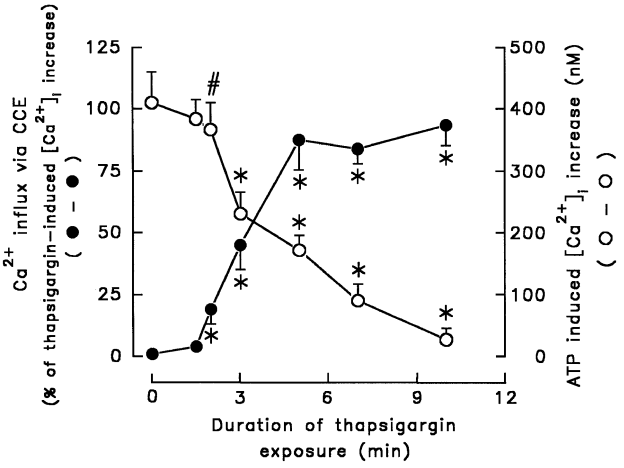

Figure 5 Effect of thapsigargin exposure time on CCE and ATP-induced $[Ca^{2+}]_i$ increase. Data were obtained from Figure 4. Thapsigargin exposure time was plotted versus thapsigargin-induced CCE or ATP-induced $[Ca^{2+}]_i$ increase. The former was expressed as the per cent of $[Ca^{2+}]_i$ decrease in response to the removal of extracellular Ca^{2+} in thapsigargin-induced $[Ca^{2+}]_i$ increase and the latter was the subsequent ATP (100 μM)-induced $[Ca^{2+}]_i$ increase in the absence of extracellular Ca^{2+}. Data were the mean ± s.d. ($n=18$). $^*P<0.001$ and $\#P<0.01$, significant different from control cells without treatment with thapsigargin, respectively.

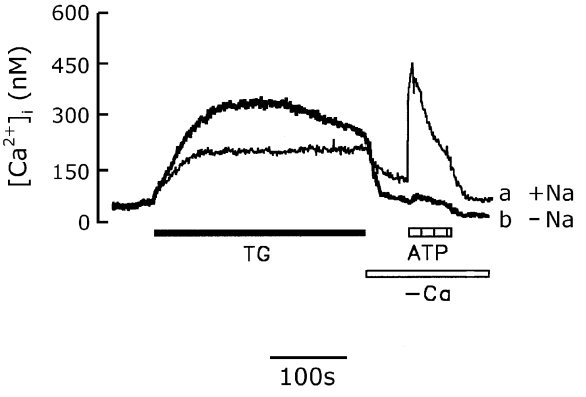

Figure 6 Effect of extracellular Na^+ on the thapsigargin-induced CCE. Cells were bathed in either Na^+-containing buffer (trace a) or Na^+-free loading buffer in which Na^+ was isotonically replaced by Tris (trace b) and the $[Ca^{2+}]_i$ changes induced by 1 μM thapsigargin (TG), Ca^{2+} removal, or 100 μM ATP were measured. The experiment was repeated five times with similar results.

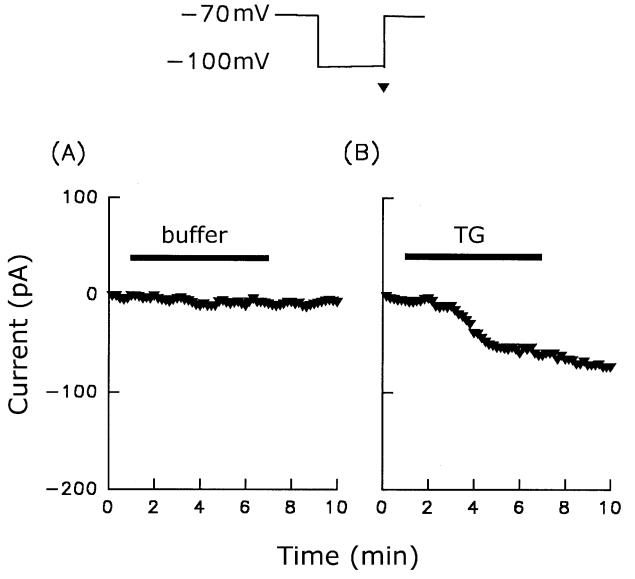

Figure 7 I_{SOC} activation by thapsigargin. The current was measured by step-pulse recording from a holding potential of −70 mV to a test potential of −100 mV over a period of 100 ms executed every 10 s. From these pulses, the amplitudes of the currents measured at −100 mV (indicated by the triangle) before, and after, perfusion with, and after washout of, buffer (A) or 1 μM thapsigargin (B) were plotted versus time. The perfusion period is indicated by the horizontal bars. Similar results were seen using six different batches of cells ($n=10$).

sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) by thapsigargin or cyclopiazonic acid (Figures 2 and 3), indicating that, under basal physiological condition, there was a dynamic equilibrium between Ca^{2+} sequestration into, and Ca^{2+} leakage from, the intracellular Ca^{2+} stores, and that no second messenger was required for Ca^{2+} leakage. Thus, by inhibiting Ca^{2+} sequestration, the Ca^{2+} stores could be depleted as a result of Ca^{2+} leakage. In the present study, two structurally distinct SERCA inhibitors, thapsigargin and cyclopiazonic acid, were equally effective in causing Ca^{2+} store depletion and increasing the Ca^{2+} permeability of the plasma membrane (Figures 2 and 3). This Ca^{2+} influx following store depletion was due to the activation of SOC since it was blocked by SK&F96365 (Figure 10).

Our results further showed that Ca^{2+} influx following store depletion was resistant to nifedipine, La^{3+} and Cd^{2+} (Figure 10). It has been previously shown that store depletion-operated current was resistant to La^{3+} in mouse anococcygeus smooth muscle cells (Wayman *et al.*, 1997) and pancreatic acinar cells (Krause *et al.*, 1996), but attenuated in A7r5 vascular smooth muscle cells (Gardner & Benoit, 2000) and guinea-pig enteric glial cells (Zhang *et al.*, 1998). Similarly, it was resistant to Cd^{2+} in mouse pancreatic acinar cells (Krause *et al.*, 1996), while being inhibited in anococcygeus smooth muscle cells, (Wayman *et al.*, 1997). Thus, different cell types may explain the variety of the cation specificity of SOC.

The K^+-driven current in response to store depletion was negligible, since KCl was replaced by CsCl in both the bathing and pipette buffers and 20 mM tetraethylammonium chloride was present. The outward rectification at the positive potential range is more prominent in the left middle panel of Figure 8. Since there was no K^+ present, the outward current induced by thapsigargin might be driven by Cl^-. In contrast, the limb of I-V curve at the negative membrane potential range is linear. Therefore, we monitored the progression of the inward current at membrane potential of −100 mV in the absence and presence of thapsigargin and expressed the time course of changes in Figure 7. When Na^+ in the bathing buffer was isotonically substituted with Tris, the thapsigargin-induced current at −100 mV was reduced (Figure 8), indicating that the contribution of Na^+ to the store depletion-induced current is profound in normal Na^+-containing physiological buffer. Thus, in rat cerebellar astrocytes, the SOC is permeable to Na^+. In contrast, it is not permeable to Sr^{2+} and Ba^{2+} (Figure 9). In mast cells

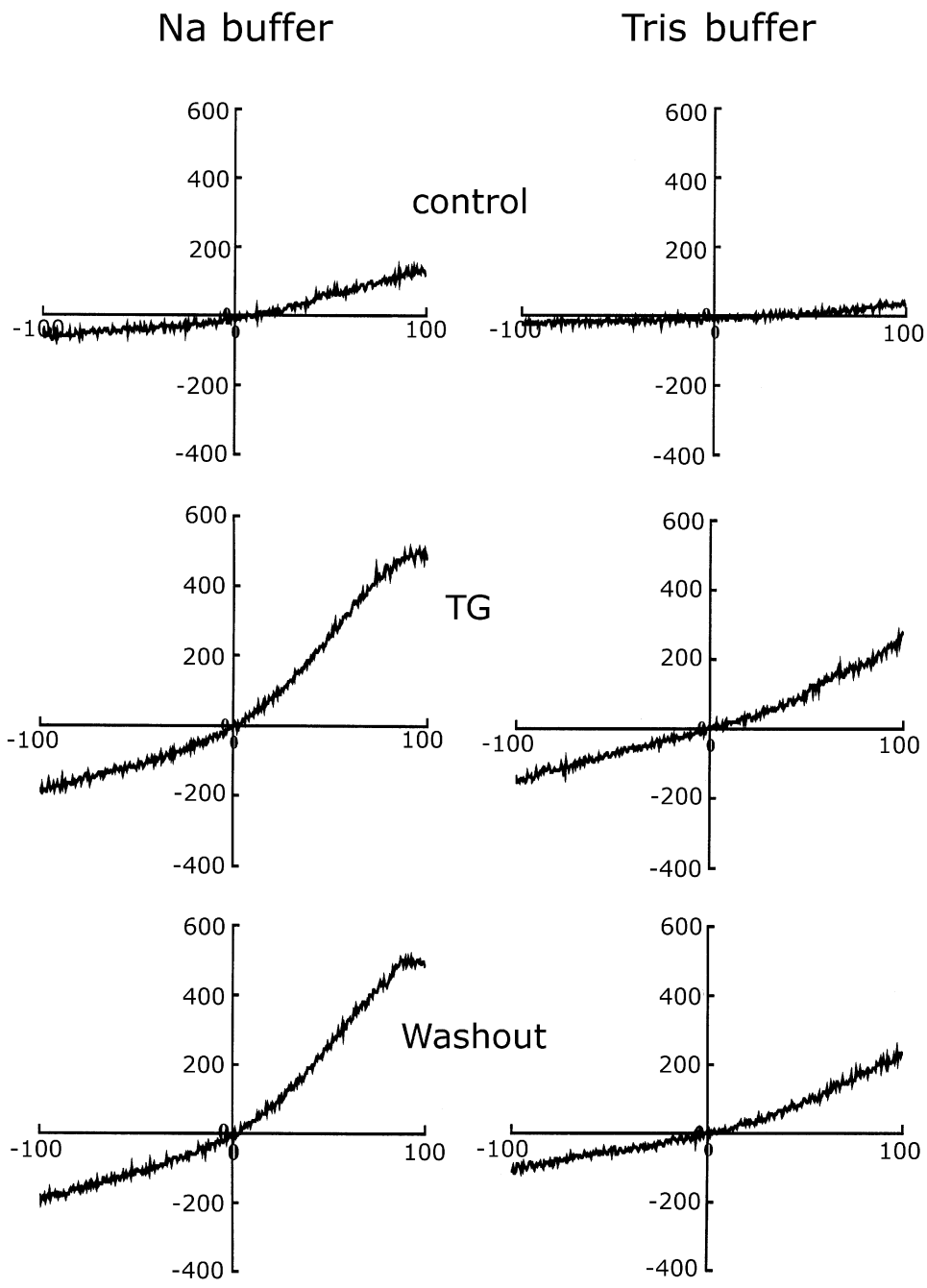

Figure 8 Membrane currents in response to extracellular application of thapsigargin. The holding potential was −40 mV and ramp pulses were given every 5 s from −100 mV to 100 mV at a speed of 2 V/s. I-V curves for control cells (top traces), cells showing the maximal response to thapsigargin (middle traces), and cells after thapsigargin washout (bottom traces) in Na^+-containing (left traces) and Na^+-free (right traces) bathing buffer are shown. Similar results were seen using six different batches of cells ($n=10$).

(Hoth & Penner, 1992) and *Xenopus* oocytes (Parekh *et al*., 1993), the SOC shows high selectivity for Ca^{2+}, while, in mouse pancreatic acinar cells, it does not discriminate between monovalent cations and possesses significant conductance for Ca^{2+} and Ba^{2+} (Krause *et al*., 1996). Different subtypes of SOC may therefore exist in different animal species.

In contrast, when the thapsigargin-induced Ca^{2+} influx was measured to reflect CCE activity, the $[Ca^{2+}]_i$ increase was higher in Na^+-free buffer than in Na^+-containing buffer (Figure 6). This finding is consistent with the hypothesis that Ca^{2+} enters through a nonselective cation channel. In the absence of Na^+, more Ca^{2+} enters cells *via* the SOC. The Na^+/Ca^{2+} exchanger is involved in regulating $[Ca^{2+}]_i$ and removal of extracellular Na^+ inhibits the activity of the exchanger, causing an increase in the $[Ca^{2+}]_i$. Thus, in our study, it is also possible that the greater thapsigargin-induced $[Ca^{2+}]_i$ increases seen in Na^+-free buffer were due to inhibition of the Na^+/Ca^{2+} exchanger. Alternatively, Na^+ may inhibit the action of thapsigargin or SOC activity.

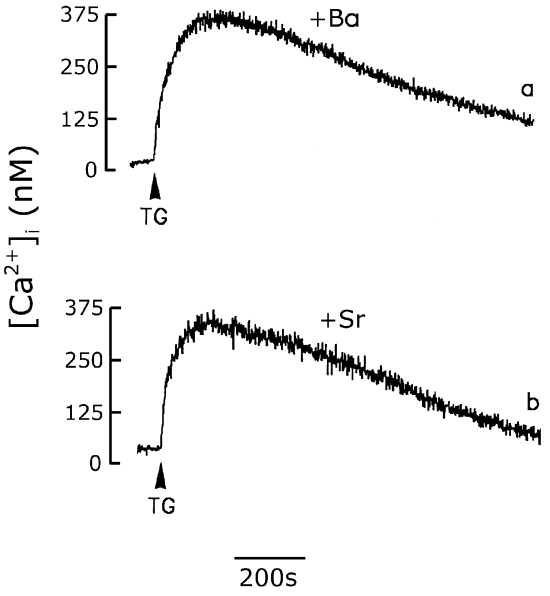

Figure 9 Effect of Ba^{2+} or Sr^{2+} on the thapsigargin-induced $[Ca^{2+}]_i$ increase. Cells were bathed in Ca^{2+}-free loading buffer in which Ca^{2+} was replaced by equimolar concentrations of Ba^{2+} (trace a) or Sr^{2+} (trace b) and the thapsigargin (1 μM)-induced $[Ca^{2+}]_i$ increase was measured. The experiment was repeated three times with similar results.

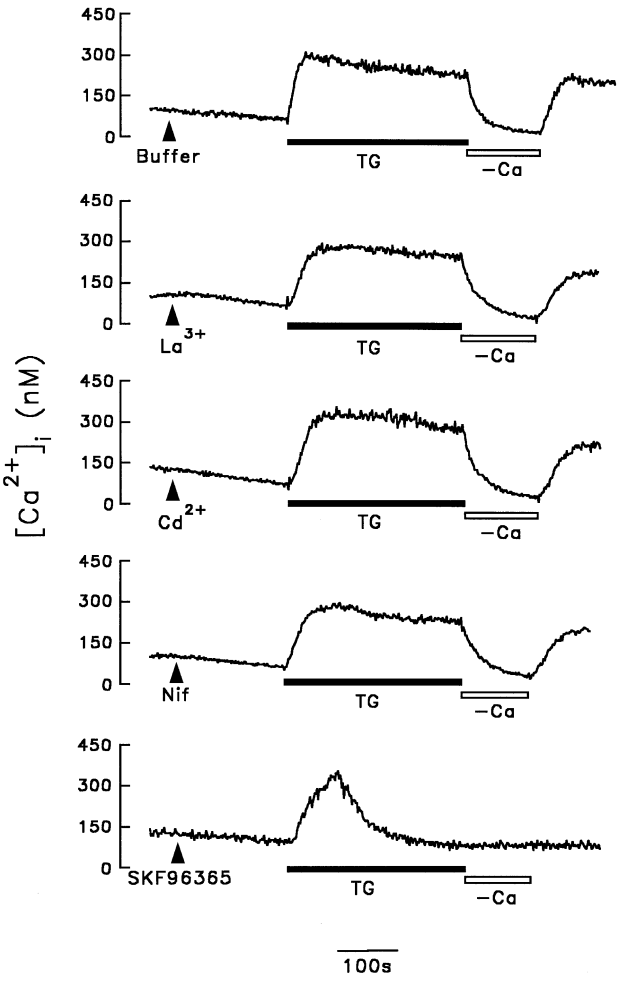

Figure 10 Effect of La^{3+}, Cd^{2+}, nifedipine or SK&F96365 on thapsigargin-induced CCE. After cells were initially treated with buffer, 100 μM La^{3+}, 100 μM Cd^{2+}, 10 μM nifedipine (NIF) or 10 μM SK&F96365 as indicated, thapsigargin (TG) (1 μM) was then added in each group. Five min after thapsigargin addition, extracellular Ca^{2+} was removed from and added back to the bathing buffer. $[Ca^{2+}]_i$ changes were measured. Similar results were seen using three different batches of cells ($n=8$).

Protein phosphorylation plays a role in CCE regulation. Firstly, protein tyrosine phosphorylation is involved in CCE. In human platelets, depletion of intracellular Ca^{2+} stores stimulates protein tyrosine phosphorylation (Vostal *et al*., 1991; Jenner *et al*., 1994), while, in human foreskin fibroblasts, bradykinin- or thapsigargin-induced Ca^{2+} entry is inhibited by tyrosine kinase inhibitors (Lee *et al*., 1993). In addition, there is increasing evidence that serine/threonine phosphorylation potentiates CCE. In *Xenopus* oocytes, okadaic acid potentiates Ca^{2+} entry once the stores have been emptied (Parekh *et al*., 1993), while, in lymphocytes and astrocytoma cells, it also prevents CIF degradation and enhances CCE (Randriamampita & Tsien, 1995). However, opposing effects of serine/threonine phosphorylation have been reported in CCE regulation in human neutrophils (Montero *et al*., 1994), platelets (Murphy *et al*., 1996), HeLa cells (Berlin & Preston, 1993), and rat basophilic leukaemia cells (BRL-2H3) (Parekh & Penner, 1995). In the present study, we showed that CCE in rat cerebellar astrocytes was regulated by phosphorylation, since inhibition of protein serine/threonine phosphorylation by staurosporine potentiated CCE, while promotion of protein serine/threonine phosphorylation by activation of protein kinase C or inhibition of protein phosphatase inhibited CCE activity (Figure 11). Many studies have shown that various Ca^{2+} transporters, including plasma membrane Ca^{2+} pump, SERCA, IP_3 receptor, SOC and VOC, are regulated by phosphorylation/dephosphorylation. In this study, we compared the $[Ca^{2+}]_i$ responses with and without thapsigargin treatment. Measurements of change of $[Ca^{2+}]_i$ reflect the final $[Ca^{2+}]_i$ after the summation of the operation of all Ca^{2+} transporters within the cell. The drugs used in this study, including staurosporine, PMA and okadaic acid may affect other Ca^{2+} transporters and result in the change of $[Ca^{2+}]_i$.

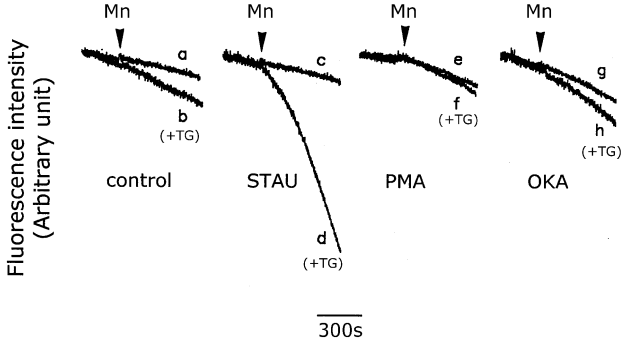

Figure 11 Effect of protein phosphorylation on the thapsigargin-induced CCE. Fura-2-loaded cells were bathed in loading buffer containing 0.2 mM Ca^{2+} alone (control) (traces a and b), or plus 100 nM staurosporine (STAU) (traces c and d), 100 nM PMA (traces e and f), or 2 nM okadaic acid (OKA) (traces g and h). Five min later, 1 mM Mn^{2+} (traces a, c, e and g) or 1 mM Mn^{2+} plus 1 μM thapsigargin (TG) (traces b, d, f and h) was added, and the fluorescence quenching due to Mn^{2+} influx measured. The experiment was repeated six times using different batches of cells with similar results ($n=15$).

However, if there would be any, the influence is the same in control and thapsigargin-treated cells.

As shown in Figure 2B, in the presence of extracellular Ca^{2+}, removal of thapsigargin had no effect on the thapsigargin-induced $[Ca^{2+}]_i$ increase once the cells had been exposed to thapsigargin for 10 min. Similarly, the thapsigargin-induced inward current was unaffected by thapsigargin washout (Figure 7). Thus, once the intracellular Ca^{2+} stores were depleted, Ca^{2+} entered *via* SOC, regardless of whether thapsigargin was present, suggesting that thapsigargin is an irreversible inhibitor. In the presence of SERCA inhibitors, the intracellular Ca^{2+} stores were maintained in a depleted state, resulting in CCE activation and the $[Ca^{2+}]_i$ remaining at a plateau (Figures 2 and 3). This result also indicates that, in rat cerebellar astrocytes, the SOC is not inhibited by a high $[Ca^{2+}]_i$. In Jurkat leukaemic T lymphocytes and rat basophilic leukaemia cells (RBL-2H3), SOCs are regulated by high $[Ca^{2+}]_i$ feedback inhibition (Zweifach & Lewis, 1995; Parekh, 1998), while, in RBL-1 cells, they are activated by a low $[Ca^{2+}]_i$ independently of global Ca^{2+} store depletion (Krause *et al*., 1999).

The SOC-induced CCE plays a crucial role in the homeostasis of $[Ca^{2+}]_i$, which in turn controls many cellular functions. In the disease states, the delicate balance of diverse signal transduction pathways might be injured and phosphorylation regulation changed. Consequently, dysregulation of $[Ca^{2+}]_i$ resulted from the disturbances of CCE will be linked to cell death or degeneration. CCE may therefore represent an important therapeutic target for the design of drugs. In cerebellar astrocytes, CCE has not been fully characterized. Whether depolarization occurs following Na^+ influx mediated by CCE requires further investigation. Similarly, the effect of this increased cytosolic Na^+ concentration on mitochondrial Ca^{2+} regulation is not clear. In addition to refilling the intracellular Ca^{2+} stores, the increased Ca^{2+} *via* SOC may also have other cellular functions. For example, Ca^{2+} in the microdomain of channel mouth may activate enzymes or target proteins. The possibility that second messengers may modulate SOCs in cerebellar astrocytes is of interest. The cerebellar astrocytes are the major arachidonic acid production site in the brain (Moore *et al*., 1991), the interrelationship of SOC and second messenger-operated Ca^{2+} entry also needs further study.

In conclusion, in rat cerebellar astrocytes, store depletion activates CCE *via* SOCs, which are permeable to Na^+, but not to Sr^{2+} and Ba^{2+}. In addition, SOC activity is regulated by serine/threonine phosphorylation.

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