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Inhibition of glutamate-induced delayed calcium deregulation by 2-APB and La^{3+} in cultured cortical neurones

Christos Chinopoulos,*,†,[1] Akos A. Gerencser,*,[1] Judit Doczi,* Gary Fiskum† and Vera Adam-Vizi*

*Semmelweis University, Department of Medical Biochemistry, Neurobiochemical Group, Hungarian Academy of Sciences, Budapest, Hungary

†University of Maryland, Department of Anesthesiology, Baltimore, Maryland, USA

Abstract

Exposure of neurones in culture to excitotoxic levels of glutamate results in an initial transient spike in $[Ca^{2+}]_i$ followed by a delayed, irreversible $[Ca^{2+}]_i$ rise governed by rapid kinetics, with Ca^{2+} originating from the extracellular medium. The molecular mechanism responsible for the secondary Ca^{2+} rise is unknown. Here, we report that the delayed Ca^{2+} entry in cortical neurones is diminished by 2-aminoethoxydiphenyl borate (2-APB: $IC_{50} = 62 \pm 9$ μM) and La^{3+} ($IC_{50} = 7.2 \pm 3$ μM), both known to inhibit transient receptor potential (TRP) and store-operated Ca^{2+} (SOC) channels. Application of thapsigargin, however, failed to exacerbate the delayed Ca^{2+} deregulation, arguing against a store depletion event as the stimulus for induction of the secondary $[Ca^{2+}]_i$ rise. In addition, these neurones did not exhibit SOC entry. Unexpectedly, application of ryanodine or caffeine significantly inhibited glutamate-induced delayed Ca^{2+} deregulation. In basal Ca^{2+} entry experiments, La^{3+} and 2-APB modulated the rapid rise in $[Ca^{2+}]_i$ caused by exposure of neurones to Ca^{2+} after pre-incubating in a calcium-free medium. This basal Ca^{2+} influx was mitigated by extracellular Mg^{2+} but not aggravated by thapsigargin, ryanodine or caffeine. These results indicate that 2-APB and La^{3+} influence non-store-operated Ca^{2+} influx in cortical neurones and that this route of Ca^{2+} entry is involved in glutamate-induced delayed Ca^{2+} deregulation.

Keywords: 2-aminoethoxydiphenyl borate, delayed Ca^{2+} deregulation, excitotoxicity, La^{3+}, store-operated calcium entry, transient receptor potential.

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Delayed calcium deregulation (DCD) is a phenomenon originally described by Manev and colleagues (Manev *et al*. 1989), further characterized by the groups of Thayer (Randall and Thayer 1992) and Tymianski (Tymianski *et al*. 1993b), addressing the latent loss of calcium homeostasis of cultured neurones upon exposure to glutamate. The phenomenon is invariably demonstrated in every neuronal cell type studied, i.e. spinal (Tymianski *et al*. 1993b), hippocampal (Randall and Thayer 1992), cerebellar granule (Budd and Nicholls 1996), striatal (Alano *et al*. 2002) and cortical neurones (Rajdev and Reynolds 1994). DCD is not observed if high extracellular K^+ is alternatively employed to elevate $[Ca^{2+}]_I$; this led to the proposal of a 'source specificity' hypothesis of Ca^{2+}-induced neurotoxicity (Tymianski *et al*. 1993b). However, this idea has been challenged by subsequent studies showing that activation of NMDA receptors produces much larger Ca^{2+} entry than activation of voltage-dependent Ca^{2+} channels by high extracellular K^+ (Hyrc *et al*. 1997). The initial spike in $[Ca^{2+}]_i$ induced by glutamate is due to opening of the ligand-gated glutamate receptors, in addition

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Address correspondence and reprint requests to Prof. Vera Adam-Vizi, Semmelweis University, Department of Medical Biochemistry, Budapest H-1444, P.O. Box 262, Hungary. E-mail: av@puskin.sote.hu

[1]Christos Chinopoulos and Akos A. Gerencser contributed equally to this work.

Abbreviations used: ANT, adenine nucleotide translocator; 2-APB, 2-aminoethoxydiphenyl borate; CCD, charged coupled device; CCE, capacitative calcium entry; CICR, Ca^{2+}-induced Ca^{2+} release; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DCD, delayed calcium deregulation; DHPG, S-3,5-dihydroxyphenylglycine; DMEM, Dulbecco's modified Eagle's essential medium; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; hTRPC3, human TRP3; IP_3, inositol trisphosphate; IP_3R, inositol trisphosphate receptor; mGluR1, metabotropic glutamate receptor 1; MIC, Mg^{2+} inhibitable cation channel; NA, numerical aperture; NMDA, *N*-Methyl-D-aspartate; NMDAR, NMDA receptor; PMNCX, plasma membrane Na^+/Ca^{2+} exchanger; PTP, permeability transition pore; RyR, ryanodine receptor; SOCE, store-operated calcium entry; SOCs, store-operated calcium channels; TRP, transient receptor potential; TTX, tetrodotoxin; VDCCs, voltage-dependent calcium channels.

to secondary activation of the voltage-dependent calcium channels (VDCCs). DCD, however, is not attributed only to events downstream of NMDA receptor activation, but also to AMPA/kainate, as well as to tetrodotoxin (TTX)-sensitive channels activated by veratridine in combination with oxidative stress (Chinopoulos *et al*. 2000) or alone (Rego *et al*. 2001). Yet, the delayed $[Ca^{2+}]_i$ rise is not inhibitable by post-glutamate addition of antagonists of NMDA or non-NMDA receptors (Manev *et al*. 1989; Tymianski *et al*. 1993a), nor by blocking voltage-dependent Ca^{2+} or Na^+ channels (Hartley and Choi 1989; Manev *et al*. 1989; Randall and Thayer 1992; Tymianski *et al*. 1993a). A number of studies have shown that DCD is associated with the loss of mitochondrial membrane potential. Therefore, the secondary rise in $[Ca^{2+}]_i$ could result from the release of calcium previously sequestered by mitochondria (Nicholls and Ward 2000). In addition, the delayed $[Ca^{2+}]_i$ rise appears to be accompanied by mitochondrial sequestration, supported by *in vivo* studies (Dux *et al*. 1987) demonstrating ultra-structural alterations of the mitochondria suggestive of pore opening. However, to consign DCD and permeability transition pore (PTP) in the pragmatic order of events is not yet feasible (Nicholls *et al*. 2003). Several lines of evidence argue against the possible contribution of a reverse function of the plasma membrane Na^+/Ca^{2+} exchanger (PMNCX) to the delayed Ca^{2+} rise: (i) NMDA-induced (but not glutamate-induced) neurotoxicity is not affected by profound hypothermia (12°C) (Tymianski *et al*. 1998), a condition that diminishes the function of the exchanger dramatically (Schellenberg and Swanson 1982); (ii) the reverse function of the exchanger is eliminated upon exposure of neurones to glutamate within 3–5 min (Yu & Choi 1997); (iii) inhibition of the exchanger by KB-R7943 unveiled a trivial role for the expression of excitotoxic injury (Hoyt *et al*. 1998). However, DCD demands the existence of a discrete pathway as it precedes, and eventually leads to, plasma membrane leakiness (Tymianski *et al*. 1993a) and cell death (Tymianski *et al*. 1993a, b; Limbrick *et al*. 1995). It is firmly established that Ca^{2+} originates from the extracellular medium (Hartley and Choi 1989; Manev *et al*. 1989; Randall and Thayer 1992; Tymianski *et al*. 1993b), but DCD is not attributed to the 'traditionally' recognized Ca^{2+} channels, such as glutamate receptor-operated or voltage-gated Ca^{2+} channels (Limbrick *et al*. 2001). Along this line, it was shown that a secondary activation of a non-selective cation conductance, termed post-exposure current (I_{pe}), is induced subsequent to excitotoxic application of NMDA to hippocampal neurones and probably accounts for the delayed Ca^{2+} rise (Chen *et al*. 1997).

We explored the possibility that DCD is mediated by transient receptor potential (TRP) channels, which are abundant in nervous tissue (Montell *et al*. 2002). Currently, a few members of the TRP family are candidates for the so-called 'store-operated Ca^{2+} entry' [(SOCE) also known as capacitative calcium entry (CCE)]. SOCE is a process whereby the depletion of intracellular calcium stores (likely endoplasmic or sarcoplasmic reticulum) activates plasma membrane Ca^{2+} permeable channels (Putney 1986). SOCE is centrally positioned among signal transduction and $[Ca^{2+}]_i$ homeostasis in both excitable and non-excitable cells (Venkatachalam *et al*. 2002). However, unequivocal evidence showing that TRP channels account for SOCE is yet to be reported (Clapham 2003). In addition, several members of the TRP channel family operate in a store-independent manner (Braun *et al*. 2001; Obukhov and Nowycky 2002; Zitt *et al*. 2002).

Our results are consistent with the hypothesis that activation of TRP channels is responsible for the delayed $[Ca^{2+}]_i$ rise. However, they do not support a role for intracellular Ca^{2+} store depletion in triggering DCD. This hypothesis is supported by the recent demonstration that TRPM7, a member of the melastatin branch of the TRP family, is responsible for neuronal death (Aarts *et al*. 2003) caused by oxygen-glucose deprivation, a model previously reported to mediate neuronal demise through NMDA receptor (NMDAR) activation (Goldberg *et al*. 1987; Goldberg and Choi 1993).

Materials and methods

Preparation of cortical neurones

Primary cultures of cortical neurones were prepared from Sprague-Dawley rats (17th day *in utero*). All animal procedures were carried out in accordance with the National Institutes of Health and the University of Maryland, Baltimore, Animal Care and Use Committee Guidelines. Neurones were grown on 25 mm coverslips for 10–16 days *in vitro*, at a density of approximately 50 000 cells/coverslip, supplemented with Dulbecco's modified Eagle's essential medium (DMEM), glutamine, neurobasal medium and B27 supplement. Glial proliferation was prevented by adding cytosine-arabinofuranoside (5 μM) 24 h after plating. Immunocytochemical measurements of glial fibrillary acid protein (GFAP) confirmed that cultures contained < 1% glia.

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

To measure $[Ca^{2+}]_i$, neurones were loaded with fura-2 AM, fura-6F AM or fura-FF AM (2 μM) at 37°C for 20 min, followed by a 10 min hydrolysis period. Single cell fluorescence of fura-2, fura-6F or fura-FF was ratio imaged by alternating excitation at 340 nm and 380 nm (Polychrome IV, Till, Munich, Germany), and emission at 510 nm. Image sequences (10 s/ratio frame, 50 ms exposure time, 2 × 2 binning) were acquired using an ORCA-ER cooled digital CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) mounted on a Nikon Eclipse TE2000-S inverted microscope (SFluor 20 × 0.75 NA and 40× 1.2 numerical apeture (NA) for $[Ca^{2+}]_i$ and $[Ca^{2+}]_{ER}$ determinations, respectively; Nikon Corp., Tokyo, Japan). Image acquisition was controlled by Metafluor 5.0 (Universal Imaging Corp., West Chester, PA, USA). The sample holder and the perfusate (50 mL/h flow rate) were temperature controlled at 37°C at the side of

the recording. The composition of the perfusate was, in mM: NaCl 120, KCl 3.5, KH_2PO_4 0.4, HEPES 20, $NaHCO_3$ 5, glucose 15, $CaCl_2$ 1.3 (or 2.6 where indicated), $MgCl_2$ 1 (or nominally Mg^{2+}-free for DCD experiments in the absence or presence of inhibitors). Whenever $LaCl_3$ was used, KH_2PO_4 and $NaHCO_3$ were excluded from the medium; control experiments verified that the lack of these chemicals did not account for the effects of $LaCl_3$.

Delayed Ca^{2+} deregulation was assayed by counting the cells on fura-6F ratio plots, which exhibited a sudden, irreversible rise in fura-6F fluorescence ratio after the initial glutamate peak. Half-times ($t_{1/2}$) of $[Ca^{2+}]_i$ decay for basal Ca^{2+} entry experiments were calculated by fitting a single-exponential decay function on each trace, from the peak in 400 s length. Image analysis was carried out in Metafluor Analyst (Universal Imaging Corp.), and data analysis in Mathematica 4.2 (Wolfram Research, Champaign, IL, USA) and SigmaPlot 8 (SPSS Inc., Chicago, IL, USA).

$[Mg^{2+}]_i$ imaging

$[Mg^{2+}]_i$ determination was performed similarly to the $[Ca^{2+}]_i$ imaging. Neurones were loaded with Mag-fura red-AM (3 μM) at 37°C for 15 min, followed by a 5 min hydrolysis period. Single-cell fluorescence of Mag-fura red was ratio imaged by alternating excitation at 430 nm and 490 nm, and emission at > 570 nm. The affinity constant (K_d) of Mag-fura red was determined in a cuvette fluorimeter (PTI Deltascan, New Brunswick, NJ, USA) and estimated to be 2.4 mM (37°C; pH 7.05). Ratios were calibrated *in vitro* using the Grynkiewicz equation (Grynkiewicz *et al*. 1985) with a measured viscosity correction (Poenie 1990). Image sequences (30 s/ratio frame, 100 ms exposure time, 2 × 2 binning) were acquired by the CCD camera. The sample holder and the perfusate (50 mL/h flow rate) were temperature controlled at 37°C at the side of the recording. The composition of the perfusate was, in mM: NaCl 120, KCl 3.5, KH_2PO_4 0.4, HEPES 20, $NaHCO_3$ 5, glucose 15, $CaCl_2$ 1.3, $MgCl_2$ 1 (or nominally Mg^{2+}-free, where indicated).

IC_{50} determination of 2-APB and La^{3+}-mediated inhibition of DCD

Cortical neurones were plated in 96-well polystyrene dishes (Costar; Sigma, St Louis, MO, USA) at the same density as for the imaging experiments. To avoid background fluorescence of polystyrene, the red fluorescent mag-fura red was used for measuring $[Ca^{2+}]_i$. Cells were loaded with the AM dye (3 μM) for 20 min, followed by a 5 min hydrolysis period. Mag-fura-red has a low affinity for Ca^{2+} (K_d = 70 μM; measured in a cuvette fluorimeter, 37°C, pH 7.05; PTI Deltascan); it is therefore only responsive to large magnitude rises in Ca^{2+}, as occur during DCD, and is not the primary response for the glutamate stimulus. Macroscopic, cumulative fluorescence of Mag-fura red was ratioed with a plate reader fluorimeter (Victor^3; Perkin Elmer, Turku, Finland) using excitation filters at 420–430 nm and 490–500 nm (Omega Optical, Brattleboro, VT, USA) and emission at 660 nm. Fluorescence background of each well was determined before loading cells with the AM dye. Inhibition of DCD was assayed by end point measurement of the background-corrected Mag-fura red ratio increase relative to the baseline after 60 min of treatment with 100 μM glutamate + 10 μM glycine in the presence of 14–300 μM 2-APB or 0.4–200 μM La^{3+} (eight concentrations performed in triplicate). Experiments were carried out in buffers similar to those used in the imaging experiments but containing 44 mM $NaHCO_3$, and cultures were kept in a CO_2 incubator at 37°C during the treatment.

Materials

2-APB, $LaCl_3$, cyclosporin A, thapsigargin, ryanodine, caffeine, 4-Br-A23187, nifedipine, cytosine-arabinofuranoside, MK-801 and CNQX were from Sigma. Fura-2 AM, fura-6F AM, fura-FF AM, mag-fura-red-AM and fura-6F5K$^+$ salt were from Molecular Probes (Eugene, OR, USA). Bongkrekic acid was from Calbiochem (EMD Biosciences, Inc., Darmstadt, Germany). Standard laboratory chemicals were from Sigma.

Results

DCD is abolished by 2-APB and La^{3+}

Cortical neurones exposed to 100 μM glutamate + 10 μM glycine exhibit an abrupt increase in $[Ca^{2+}]_i$ attributed to activation of ligand-gated glutamate receptors (Fig. 1a, 100 s, Table 1). Although glutamate and glycine remain in the perfusate throughout the experiment, $[Ca^{2+}]_i$ plateaus at a level lower than the ligand-induced peak, due to desensitization of the NMDA receptors (Mayer and Westbrook 1985), as well as inhibition of the receptor by elevated $[Ca^{2+}]_i$ (Legendre *et al*. 1993). After ≥ 5 min, and over a period of 60 min, neurones lose the ability to maintain $[Ca^{2+}]_i$ at the newly established level and exhibit asynchronous, large and abrupt increases in fura-6F fluorescence ratio. Fura-6F has a relatively high K_d (2.47 μM) for Ca^{2+} and it measures $[Ca^{2+}]$ reliably in the range of 0.5–50 μM (Chinopoulos *et al*. 2003). Therefore, the dye is not saturated at the glutamate-induced Ca^{2+} spike, a phenomenon observed with high-affinity calcium indicators (Stout and Reynolds 1999). This also 'unmasks' the magnitude of the secondary Ca^{2+} rise. It is apparent that the majority of neurones undergo DCD, with a mean onset t_{mean} = 910 ± 160 s after application of the glutamate stimulus (Table 1). While there is strong evidence that DCD is due to plasmalemmal Ca^{2+} influx (see introduction), the extent of the burden that it imposes on calcium extrusion (e.g. plasma membrane Ca^{2+} ATPase) and sequestering mechanisms (e.g. mitochondrial calcium uptake) has not yet been clarified. Therefore, in a separate set of experiments, $[Ca^{2+}]_e$ was removed from the perfusate by switching to a nominally Ca^{2+}-free medium plus 25 μM EGTA at 1500 s, which prevented further occurrence of DCD (Fig. 1b) and caused a decline of $[Ca^{2+}]_i$ in cells that were already deregulated. The slope of this decline was determined for each deregulated cell and was plotted against the time elapsed from the onset of DCD to the time of $[Ca^{2+}]_e$ washout (Fig. 1c). Linear regression analysis of this plot showed that removal of $[Ca^{2+}]_e$ shortly after the onset of DCD causes a rapid decrease in $[Ca^{2+}]_i$ but that subsequently, neurones lose the ability to rapidly restore resting $[Ca^{2+}]_i$. This finding raises the possibility that the delayed Ca^{2+} influx

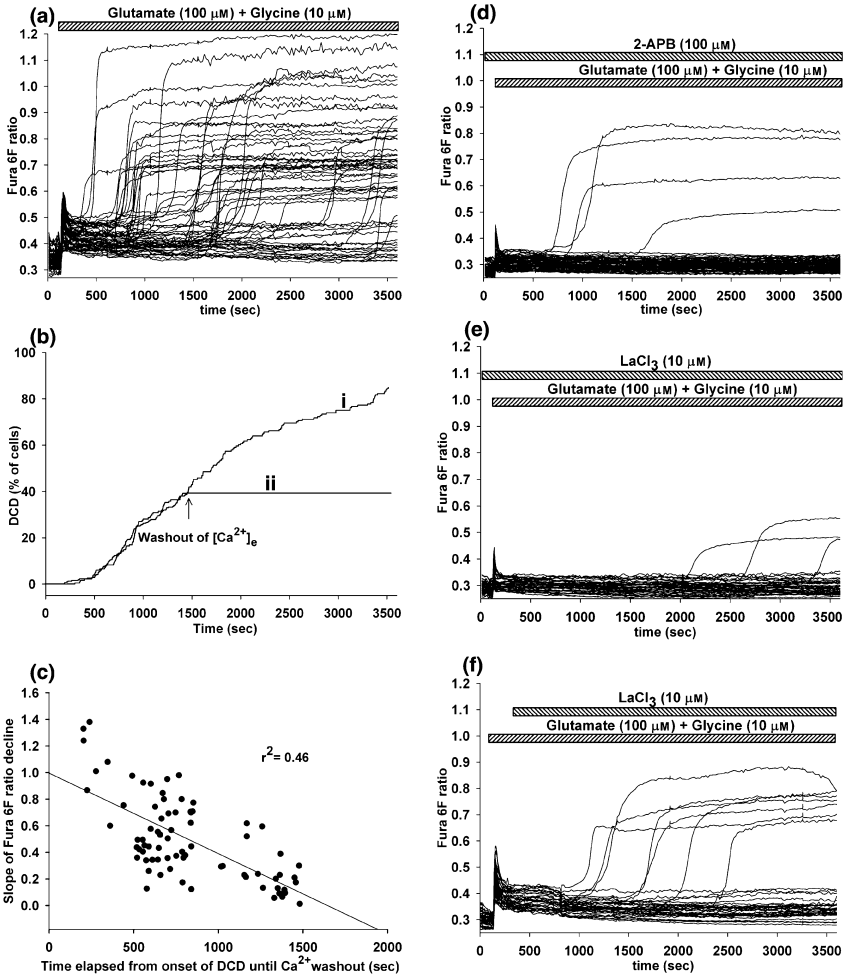

Fig. 1 DCD induced by glutamate (100 μM) plus glycine (10 μM) in cortical neurones and the effect of 2-APB and La^{3+}. a, d, e, f: Fura-6F ratio fluorescence of $[Ca^{2+}]_i$ upon exposure to glutamate plus glycine in the presence and absence of 2-APB (100 μM) or $LaCl_3$ (10 μM) recorded for 1 h. Glutamate plus glycine were added to the perfusion medium at 100 s and were present throughout the entire experiment. (a) No inhibitor present. Traces are representative of six independent experiments. 2-APB (d) or La^{3+} (e) were given 10 min prior to glutamate plus glycine exposure and were present throughout the entire experiment. Traces are representative of five (d) or three (e) independent experiments. (f) Effect of $LaCl_3$ added 300 s after the addition of glutamate plus glycine (representative of three independent experiments). (b) Percentage of cells that underwent DCD upon exposure to glutamate plus glycine (added at 100 s) versus time. Trace (i) was calculated from the data set corresponding to Table 1. In trace (ii) the perfusion was switched to Ca^{2+}-free medium + 25 μM EGTA containing glutamate plus glycine at 1500 s. (c) Linear regression analysis of slope of fura-6F ratio fluorescence (10^{-3} ratio units/s) versus time elapsed from DCD onset until removal of extracellular calcium, in s. Data pooled from three independent experiments.

elicits a sequence of events that gradually incapacitate calcium extrusion/sequestering mechanisms.

2-APB (100 μM) added 10 min prior to exposure to glutamate led to a dramatic decrease in the incidence of the secondary $[Ca^{2+}]_i$ rise (Fig. 1d, Table 1). The same phenomenon was observed if 10–100 μM $LaCl_3$ was applied, but 1 μM $LaCl_3$ was ineffective (Fig. 1e, Table 1). In an effort to determine the IC_{50} for 2-APB and La^{3+}, DCD was assayed by measuring the cumulative response of mag-fura red (used as a low affinity Ca^{2+} dye) -loaded cortical neurones cultured in 96-well dishes. Parallel imaging experiments using Mag-fura red and the standard DCD protocol (100 μM glutamate + 10 μM glycine for 1 h) were performed (not shown). Due to the low affinity ($K_d = 70$ μM) of Mag-fura red, the difference between the fluorescence ratio before, and 60 min after the addition of glutamate plus glycine reflects the fraction of cells that underwent DCD. 2-APB (14–300 μM) applied 10 min before, and La^{3+} (0.4–200 μM) applied together with glutamate, dose-dependently diminished the ratio increase evoked by the glutamate treatment. IC_{50} of 2-APB inhibition on DCD was estimated to be 62 ± 9 μM ($n = 4$ plates), whereas that for La^{3+} was 7.2 ± 3 μM ($n = 3$ plates).

Although $LaCl_3$ blocks voltage-dependent Ca^{2+} channels (Nelson *et al*. 1984), this pathway has previously been excluded as a candidate for the secondary $[Ca^{2+}]_i$ rise (see introduction). In addition to inhibiting DCD, 100 μM 2-APB and 10 μM $LaCl_3$ reduced the glutamate-induced $[Ca^{2+}]_i$ peak (Figs 1d and e, Table 1) and accelerated the decay to a newly established $[Ca^{2+}]_i$ plateau. The latter observation is similar to that of Baba and colleagues (Baba *et al*. 2003). However, in contrast to our results with glutamate, these authors did not detect an inhibitory effect of 2-APB or La^{3+} on the peak amplitude of the NMDA-induced $[Ca^{2+}]_i$ transients. This reflects the possibility that glutamate-induced non-NMDAR $[Ca^{2+}]_i$ influx encompasses 2-APB and La^{3+}-sensitive targets. Application of a lower concentration (30 μM) of 2-APB did not result in a decrease in glutamate-induced $[Ca^{2+}]_i$ peak, whereas the incidence of DCD was slightly decreased with an increase in the mean onset time of DCD (t_{mean}; Table 1). As Baba and colleagues demonstrated

Table 1 Characteristics of glutamate-induced DCD in the presence and absence of various compounds

	n	Total number of cells	DCD % of cells	Onset of DCD t_{mean} (s)	Baseline $[Ca]_i$ (fura-6F ratio fluorescence)	Glutamate + glycine induced $[Ca]_i$ peak (fura-6F ratio at 100 s, absolute value)
Control	6	332	84.5 ± 9.7	910 ± 160	0.31 ± < 0.001	0.397 ± < 0.001
2-APB (100 μM)	5	490	4.5 ± 1.0*[a]	917 ± 149 [n.s]	0.30 ± < 0.001	0.354 ± < 0.001*[b]
2-APB (30 μM)	3	189	77.8 ± 4.8*[a]	1366 ± 125*[b]	0.30 ± < 0.001	0.398 ± < 0.001 [n.s]
$MgCl_2$ (10 mM) (pre-incubation)	5	603	8.2 ± 3.0*[a]	894 ± 188 [n.s]	0.31 ± < 0.001	0.370 ± < 0.001 [n.s]
$LaCl_3$ (1 μM)	3	155	70.0 ± 6.9*[a]	902 ± 155 [n.s]	0.30 ± < 0.001	0.378 ± < 0.001 [n.s]
$LaCl_3$ (10 μM)	3	168	8.1 ± 1.2*[a]	2480 ± 182*[b]	0.30 ± < 0.001	0.357 ± < 0.001*[b]
$LaCl_3$ (10 μM) (after glutamate)	3	151	9.8 ± 3.2*[a]	1654 ± 111*[b]	0.30 ± < 0.001	0.399 ± < 0.001 [n.s]
$LaCl_3$ (100 μM)	3	199	2.02 ± 1.33*[a]	2607 ± 188*[b]	0.31 ± < 0.001	0.366 ± < 0.001 [n.s]
Ryanodine (1 μM)	3	204	28.4 ± 0.6*[a]	1816 ± 173*[b]	0.30 ± < 0.001	0.383 ± < 0.001 [n.s]
Caffeine (10 mM)	3	248	7.67 ± 1.7*[a]	1450 ± 292*[b]	0.30 ± < 0.001	0.368 ± < 0.001 [n.s]
SK&F 96365 (5 μM)	3	255	88.9 ± 10.4 [n.s]	780 ± 169 [n.s]	0.30 ± < 0.001	0.394 ± < 0.001 [n.s]
Cyclosporin A (1 μM)	3	198	86.7 ± 12.8 [n.s]	885 ± 168 [n.s]	0.31 ± < 0.001	0.431 ± 0.008*[b]
Bongkrekic acid (20 μM)	3	178	89.5 ± 13.9 [n.s]	931 ± 174 [n.s]	0.33 ± < 0.001	0.467 ± 0.009*[b]
Thapsigargin (1 μM)	3	158	87.1 ± 9.8 [n.s]	911 ± 160 [n.s]	0.30 ± < 0.001	0.404 ± < 0.001 [n.s]

2-APB, $LaCl_3$, ryanodine, caffeine, SK&F 96365, cyclosporin A, bongkrekic acid and thapsigargin were present for 10–30 min (as described in the text) prior to exposure to glutamate plus glycine and throughout the entire duration of the experiment. $MgCl_2$ was present only for 30 min prior to exposure to glutamate plus glycine. $LaCl_3$, where indicated, was applied 300 s after perfusion with glutamate plus glycine ($LaCl_3$ 10 μM after glutamate). n = number of independent experiments. Statistics: Mann–Whitney rank sum test.

[a]Significant compared to control, $p < 0.001$, [b]significant compared to control, $p < 0.005$, n.s: not significant compared to control, $p < 0.005$.

electrophysiologically that 2-APB and La^{3+} do not inhibit the NMDA receptor, our results are not attributable to a confounding inhibitory action on the NMDA receptor itself. Nevertheless, the moderately reduced glutamate-induced $[Ca^{2+}]_i$ peak and the accelerated return towards baseline values (Figs 1d and e, Table 1) may contribute to the diminished incidence and mean onset time of DCD by other means. Therefore, we applied the compounds 300 s after the start of perfusion with glutamate. It is apparent from Fig. 1(f) and Table 1 that application of $LaCl_3$ (10 μM) strongly inhibited DCD while the glutamate-induced $[Ca^{2+}]_i$ peak remained unchanged. The protective effect of 2-APB given after glutamate + glycine was, however, less pronounced, diminishing the incidence of DCD to approximately 55% (not shown).

Mitochondrial PTP inhibitors fail to ameliorate DCD

To address the role of PTP in our system, cortical neurones were treated with cyclosporin A (1 μM) for 30 min prior to exposure to glutamate; the inhibitor was also present in the perfusate for the entire duration of the experiment. As shown in Fig. 2(a) and Table 1, there was no statistical difference in the percentage of cells undergoing DCD, or in the mean time of onset in the presence or absence of cyclosporin A. It is noteworthy that DCD is an event usually demonstrated in the absence of Mg^{2+} to relieve the block of the NMDA receptor, and it has been shown that Mg^{2+} is critical for conferring cyclosporin A sensitivity of the permeability transition in rat liver mitochondria (Andreyev *et al*. 1994). In an effort to increase $[Mg^{2+}]_i$, neurones were pre-treated with 10 mM $MgCl_2$ for 30 min prior to glutamate exposure without cyclosporin A. This led to an increase in the baseline $[Mg^{2+}]_i$ from 0.65 ± 0.02 mM to 1.15 ± 0.03 mM, persisting for at least 1 h irrespective of perfusing with a $MgCl_2$-free buffer during the experiment. This regime resulted in a substantial reduction in the incidence of DCD without affecting the glutamate-induced $[Ca^{2+}]_i$ peak (Table 1). However, the exact mechanism underlying this protective effect was not further investigated. As with cyclosporin A, bongkrekic acid (20 μM) failed to decrease the incidence or the onset time of DCD, irrespective of whether it was present throughout the experiment (Fig. 2b, Table 1) or present only during a 10 min pre-incubation period (not shown). Notably, application of either cyclosporin A or bongkrekic acid led to a statistically significant increase in the glutamate-induced $[Ca^{2+}]_i$ peak (Figs 2a and 2b, respectively, and Table 1). The mechanism(s) for this peculiarity was not further investigated here.

Do cortical neurones exhibit store-operated Ca^{2+} entry?

We first investigated 'basal' Ca^{2+} entry, in which neurones were deprived of extracellular calcium ('Ca^{2+}-free' medium in the absence of Sarcoplasmic Reticulum Ca^{2+} ATPase (SERCA)

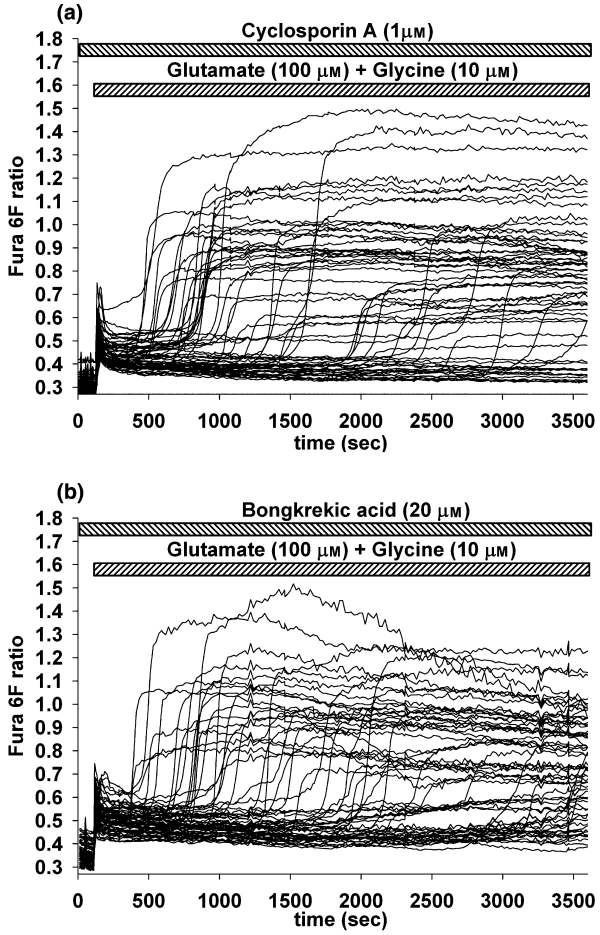

Fig. 2 Lack of effect of cyclosporin A and bongkrekic acid on glutamate induced DCD. Fura-6F ratio fluorescence of $[Ca^{2+}]_i$ of cortical neurones upon exposure to glutamate (100 μM) plus glycine (10 μM) is shown in the presence of cyclosporin A (1 μM) or bongkrekic acid (20 μM) recorded for 1 h. (a) Cyclosporin A was given 30 min prior to glutamate plus glycine exposure, and was present throughout the entire experiment. Traces are representative of three independent experiments. (b) Bongkrekic acid was present for 10 min prior to glutamate plus glycine exposure, as well as throughout the entire experiment. Traces are representative of three independent experiments.

inhibitors, containing 7.44 μM free Ca^{2+} measured by fura-6F $5K^+$ salt) for 8–10 min, followed by re-introduction of the cation to the perfusing medium. Prior to this, in order to demonstrate that the intracellular stores do contain Ca^{2+}, neurones were perfused with either Ca^{2+}-free medium (Fig. 3a, trace i), Ca^{2+}-free medium containing EGTA (25 μM) (Fig. 3a, trace i), Ca^{2+}-free medium plus thapsigargin (Tg; 1 μM) (Fig. 3a, trace iii), or Ca^{2+}-free medium containing EGTA plus Tg (Fig. 3a, trace iv). $[Ca^{2+}]_{ER}$ was estimated from the perinuclear fluorescence of fura-FF (Csordas and Hajnoczky 2001) under resting conditions when $[Ca^{2+}]_i$ is low. Therefore, the low affinity Ca^{2+} dye is unresponsive to changes in $[Ca^{2+}]_i$, but responsive to alterations in $[Ca^{2+}]$ in compartments where $[Ca^{2+}]$ is sufficiently high, such as the endoplasmic reticulum (ER). High magnification imaging revealed a perinuclear patchy pattern of higher ratios. In the presence of Tg ± EGTA, the fura-FF ratio dropped quickly (in 2–400 s) over these regions, indicating that the measured signal (at least partially) originates from the ER. In contrast, perfusing with Ca^{2+}-free medium did not cause a significant ratio change, but when EGTA was also present, a small and delayed decrease in the fura-FF ratio fluorescence was observed. To address the degree of the intracellular stores depletion upon Tg treatment, neurones were challenged with a cocktail consisting of 4-Br A23187 (10 μM), monensin (10 μM), nigericin (10 μM) and gramicidin (5 μM), applied at 200 s (trace v), or at 700 s (Fig. 3a, trace iv). It is apparent that 10 min of perfusing with EGTA + Tg depletes Tg-sensitive intracellular stores (compare trace iv with trace v at 800–900 s). To examine further the filling status of the intracellular stores, $[Ca^{2+}]_i$ was measured using fura-2 in neurones which were perfused with Ca^{2+}-free medium + EGTA ± Tg for various time intervals, followed by addition of the calcium ionophore 4-Br-A23187 (5 μM) (Fig. 3b). Under this condition, Ca^{2+} released exclusively from intracellular compartments would increase fura-2 fluorescence. Specifically, perfusion with Ca^{2+}-free medium + EGTA led to a moderately diminished response to ionophore added at 400 s (Fig. 3b, trace iv) compared with 100 s (Fig. 3b, trace ii) (0.24 ± 0.03 vs. 0.62 ± 0.03 slope ratio/min, significant, Mann–Whitney rank sum test, $p < 0.005$, 0.58 ± 0.03 vs. 0.70 ± 0.01 peak fura-2 ratio, significant, Mann–Whitney rank sum test, $p < 0.005$). When the perfusate was changed from Ca^{2+}-containing (1.3 mM) to Ca^{2+}-free medium + EGTA (< 100 nM $[Ca^{2+}]_e$) without any pre-incubation delay (Fig. 3b, trace i), the $[Ca^{2+}]_i$ transients elicited by the addition of ionophore were not different from those observed following a 100 s pre-incubation time (Fig. 3b, trace ii). Pre-treatment of the cultures with Tg (1 μM) prior to the addition of the ionophore failed to induce further increase in $[Ca^{2+}]_i$ (Fig. 3b, trace iii, 100–400 s). Application of Tg to neurones perfused with a medium containing 1.3 mM $CaCl_2$ also failed to elevate fura-2 fluorescence (not shown). Moreover, the presence of Tg did not alter significantly the $[Ca^{2+}]_i$ transients caused by addition of ionophore when cells were perfused with the EGTA-containing medium (Fig. 3b, trace iv vs. trace iii, not significant). The lack of effect of Tg is not inconsistent with the observation shown in Fig. 3a, where changes in the $[Ca^{2+}]_{ER}$ were estimated. 4Br-A23187 releases Ca^{2+} from all internal stores. Although the rise in fura-2 ratio elicited by the ionophore in the absence or presence of Tg pre-treatment was very similar, this could be explained by the existence of a relatively large Tg-insensitive compared with Tg-sensitive Ca^{2+} store. Collectively, these observations led us to conclude that Tg-sensitive intracellular stores in cultured cortical neurones contain releasable, sequestered Ca^{2+}. Perfusing the cells with Ca^{2+}-free medium does not lead to an alteration of their filling state, while chelating extracellular Ca^{2+} slightly depletes the stores,

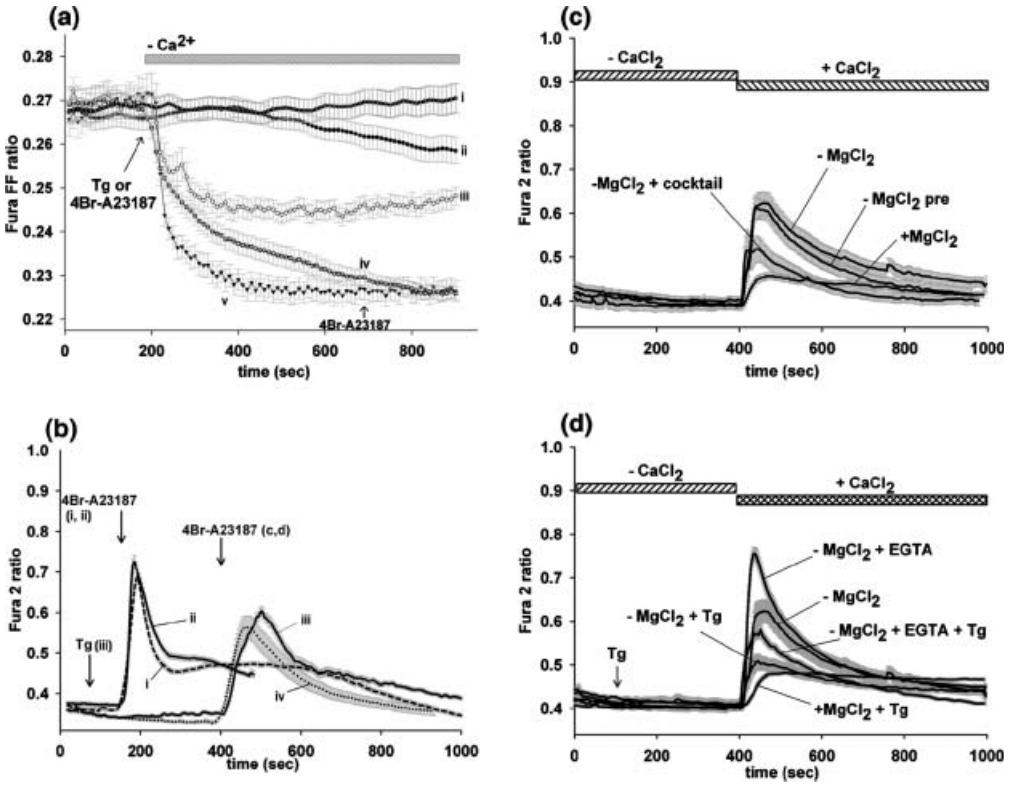

Fig. 3 (a) Effect of removal of extracellular Ca^{2+} on neuronal stores. $[Ca^{2+}]_{ER}$ was estimated as the perinuclear fluorescence ratio of fura-FF. Perfusion of neurones with Ca^{2+}-free medium (trace i), Ca^{2+}-free medium containing 25 μM EGTA (trace ii), Ca^{2+}-free medium plus 1 μM Tg (trace iii), or Ca^{2+}-free medium containing 25 μM EGTA plus 1 μM Tg (trace iv), Ca^{2+}-free medium containing 25 μM EGTA plus an ionophore cocktail consisting of 4-Br A23187 (10 μM), monensin (10 μM), nigericin (10 μM) and gramicidin (5 μM) (trace v), was started at 200 s as indicated. In trace (iv) the ionophore cocktail was applied at 700 s. Each trace represents mean ± SEM of cells from three independent experiments. (b) Neurones were perfused with Ca^{2+}-containing medium for 100 s (trace i), Ca^{2+}-free medium + EGTA for 100 s (trace ii) and 400 s (traces iii and iv). After these intervals, 5 μM 4-Br A23187 was added in the presence of EGTA. In trace (iii), Tg (1 μM) was added to the perfusate at 50 s. When 1.3 mM $CaCl_2$ was present throughout the entire duration of the experiment, fura-2 ratio plateaued at about 2.7 at 400 s (omitted from the graph). Traces are representative of three independent experiments. (c) Fura-2 ratio fluorescence of $[Ca^{2+}]_i$ of cortical neurones on basal Ca^{2+} entry. Cortical neurones were incubated in Ca^{2+}-free medium for 8–10 min (including the initial 400 s recording interval). $CaCl_2$ (2.6 mM) was re-introduced to the perfusing medium at 400 s. $-MgCl_2$ indicates absence of extracellular $MgCl_2$. + $MgCl_2$ signifies the presence of 1 mM $MgCl_2$ in the perfusate. $MgCl_2$ pre indicates that neurones were pre-exposed to 10 mM $MgCl_2$ for 30 min, but it was absent from the perfusate. Cocktail consisted of 10 μM MK-801, 10 μM CNQX and 1 μM nifedipine. The decay of $[Ca^{2+}]_i$ after the peak Ca^{2+} entry was quantified by the half-time ($t_{1/2}$ in s): $-MgCl_2$: 93 ± 11, $-MgCl_2$ + cocktail: 92 ± 12, + $MgCl_2$: 154 ± 40*, $MgCl_2$ pre: 96 ± 12. Traces are representative of three independent experiments. (d) Basal Ca^{2+} entry as measured by fura-2 ratio fluorescence of $[Ca^{2+}]_i$ in the presence and absence of Tg (1 μM). Cortical neurones were incubated in the absence of extracellular Ca^{2+} ± Tg for 8–10 min, in the presence and absence of EGTA. $CaCl_2$ (2.6 mM) was re-introduced to the perfusing medium at 400 s. $t_{1/2}$: $-MgCl_2$ + Tg: 134 ± 25*. $t_{1/2}$: $-MgCl_2$ + EGTA + Tg: 145 ± 13*, $t_{1/2}$: $-MgCl_2$ + EGTA: 84 ± 6*. Traces are representative of four independent experiments. One-way ANOVA, Tukey's post hoc analysis; *significant, compared to $-MgCl_2$, $p < 0.05$.

and a complete depletion can be achieved by inhibiting Ca^{2+} uptake through the SERCA pump.

Subsequently, basal Ca^{2+} entry was assessed by perfusing neurones with Ca^{2+}-free medium, followed by re-introduction of $[Ca^{2+}]_e$. It is apparent from Fig. 3(c) that re-introduction of Ca^{2+} to the medium induces a small increase in $[Ca^{2+}]_i$ in the presence of 1 mM extracellular Mg^{2+}, and a slow return towards basal $[Ca^{2+}]_i$ levels. DCD experiments were performed in the absence of Mg^{2+} in order to relieve the block of the NMDA receptor, otherwise the initial glutamate-induced $[Ca^{2+}]_i$ peak is severely blunted and DCD does not manifest itself. Therefore, basal Ca^{2+} entry was also investigated in the absence of extracellular Mg^{2+}. As shown in Fig. 3(c), the lack of Mg^{2+} in the medium led to a more robust increase in fura-2 ratio upon re-addition of Ca^{2+}. However, omission of extracellular Mg^{2+} may sensitize the NMDA receptor to activation. In addition, non-NMDARs and VDCCs, in a concerted action, could contribute to this Ca^{2+} influx related to the status of the plasma membrane potential, possibly affected by omission/re-introduction of

extracellular Ca^{2+}. Inclusion of a cocktail comprising the NMDA inhibitor MK-801 (10 μM), the AMPA/kainate inhibitor CNQX (10 μM) and the VDCC blocker nifedipine (1 μM) caused a reduction of the basal Ca^{2+} entry observed in the absence of Mg^{2+} (Fig. 3c). However, the peak of the $[Ca^{2+}]_i$ increase was still higher than when extracellular Mg^{2+} was present (fura-2 ratio = 0.21 ± 0.05 relative to baseline vs. 0.07 ± 0.01 in the presence of $MgCl_2$). These observations indicate that there is a basal Ca^{2+} entry in cultured cortical neurones that is unmasked in the absence of extracellular Mg^{2+}, which is influenced by, but not due to ligand and/or voltage-gated Ca^{2+} channels.

In order to investigate the spatial role of Mg^{2+} in the alleviation of basal Ca^{2+} entry, neurones were incubated in the presence of 10 mM $MgCl_2$ for 30 min. This treatment caused a rise in $[Mg^{2+}]_I$, from 0.65 ± 0.02 mM to 1.15 ± 0.03 mM, that persisted for at least 1 h even when the extracellular medium was subsequently switched to the $MgCl_2$-free perfusate. Pre-treatment of the cells in the absence of $[Mg^{2+}]_e$ (for 30 min) did not cause a significant drop in $[Mg^{2+}]_i$ (not shown). These neurones did not exhibit a significantly different peak amplitude of basal Ca^{2+} entry (0.33 ± 0.03 vs. 0.31 ± 0.03 in the presence of $MgCl_2$, one-way ANOVA Tukey's *post hoc* analysis).

Next, we investigated whether abolition of Ca^{2+} uptake by the SERCA pump using Tg (1 μM) results in capacitative calcium entry (CCE). Neurones were perfused with Ca^{2+}-free medium ± EGTA (25 μM) for 5–8 min, followed by addition of Tg, which failed to induce any change in $[Ca^{2+}]_i$ (Fig. 3d, 100–400 s). Re-introduction of extracellular Ca^{2+} caused a *decreased* Ca^{2+} entry compared with that observed in the absence of Tg, whether neurones were perfused with Ca^{2+}-free medium (fura-2 ratio = 0.11 ± 0.02 relative to baseline vs. 0.31 ± 0.03 in the absence of Tg) or with Ca^{2+}-free medium + EGTA (fura-2 ratio = 0.19 ± 0.01 relative to baseline vs. 0.36 ± 0.01 in the absence of Tg). This counterintuitive response contrasting the 'authentic' capacitative Ca^{2+} entry described for non-excitable as well as several excitable cell models (Elliott 2001; Putney 2003) is inconsistent with the possibility that in cortical neurones, depletion of Tg-sensitive Ca^{2+} stores activates Ca^{2+}-permeable non-voltage-gated cation channels located in the plasma membrane that mediate SOCE in other cell types. In support of this, the presence of a Ca^{2+} entry pathway in sympathetic, sensory and hippocampal neurones, distinct from voltage-dependent Ca^{2+} channels, was demonstrated and regulated by ryanodine-sensitive Ca^{2+} stores (Friel and Tsien 1992). Therefore, we examined the effect of ryanodine receptor agonists on Ca^{2+} entry.

Ryanodine receptor activation does not potentiate basal Ca^{2+} entry

Cortical neurones were perfused with Ca^{2+}-free medium for 5 min, followed by addition of ryanodine (1 μM) (Fig. 4a, 100–400 s) or caffeine (10 mM) (Fig. 4b, 100–400 s). Ryanodine or caffeine alone did not result in a measurable increase in $[Ca^{2+}]_i$. In addition, application of caffeine caused a minor decrease in baseline Fura 2 fluorescence. Ryanodine failed to potentiate basal Ca^{2+} entry, though it led to a slightly accelerated decay of $[Ca^{2+}]_i$ towards baseline values (Fig. 4a). The Ca^{2+} influx was strongly alleviated by extracellular Mg^{2+} and moderately decreased by concomitant inhibition of NMDARs/non-NMDARs/VDCCs by the cocktail (Fig. 4a). Likewise, caffeine did not lead to augmentation of basal Ca^{2+} entry and its effect on hastening $[Ca^{2+}]_i$ decay was not significant. These results do not support SOCE regulation by ryanodine-sensitive stores in cortical neurones.

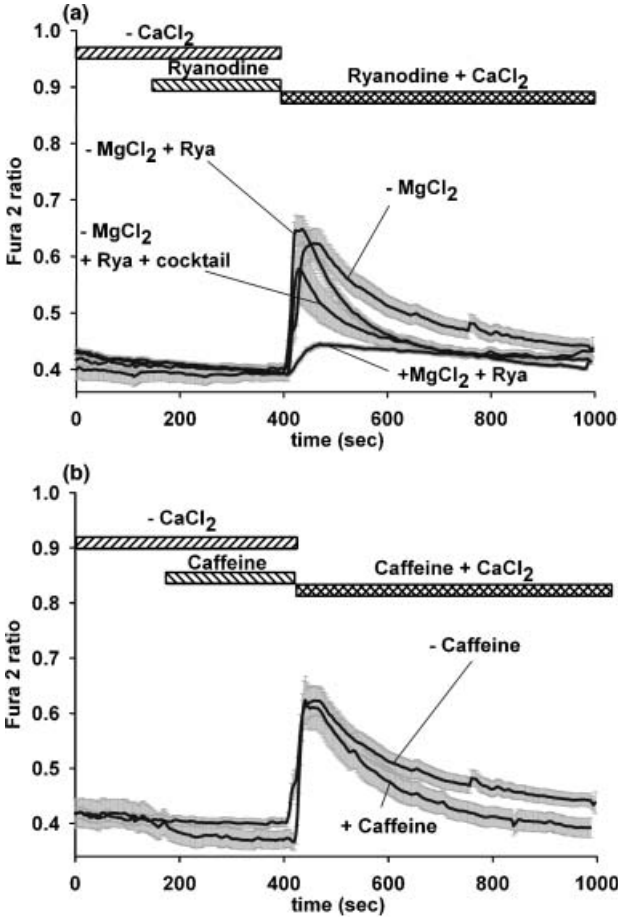

Fig. 4 Fura-2 ratio fluorescence of $[Ca^{2+}]_i$ of cortical neurones on Ca^{2+} entry in the presence of ryanodine 1 μM (a) or caffeine 10 mM (b). Cortical neurones were incubated in Ca^{2+}-free medium for 8–10 min (including the initial 400 s recording interval). $CaCl_2$ (2.6 mM) was re-introduced to the perfusing medium at 400 s. $-MgCl_2$ signifies absence of extracellular $MgCl_2$. + $MgCl_2$ signifies presence of 1 mM $MgCl_2$ in the perfusate. Rya: ryanodine. $t_{1/2}$: $-MgCl_2$ + Rya: 64 ± 4*, $t_{1/2}$: $-MgCl_2$ + Rya + cocktail: 57 ± 13*. + caffeine: 94 ± 10. Graphs are representative of three independent experiments. One-way ANOVA, Tukey's post hoc analysis; *significant, compared to Fig. 3(c) $-MgCl_2$, $p < 0.05$.

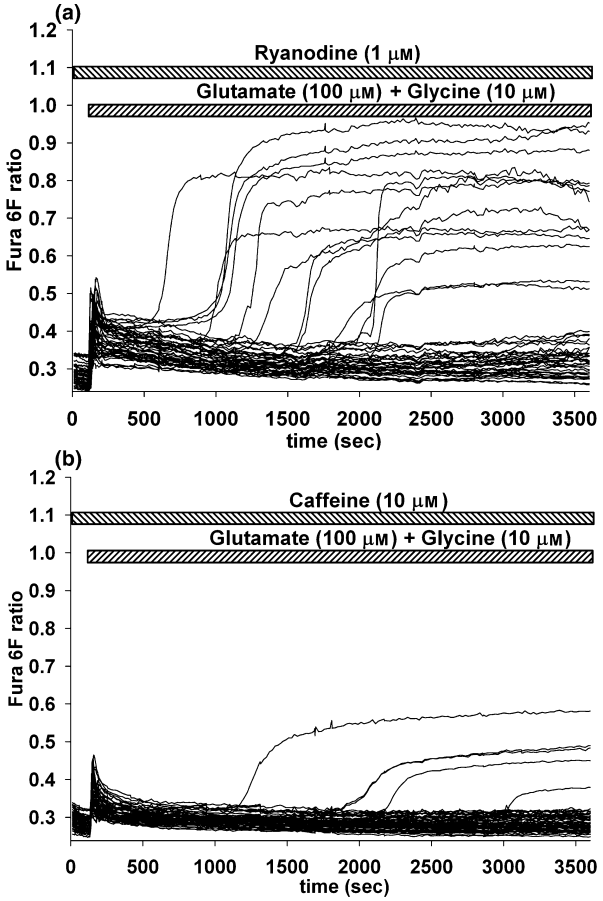

Fig. 5 Fura-6F ratio fluorescence of $[Ca^{2+}]_i$ of cortical neurones upon exposure to glutamate (100 μM) plus glycine (10 μM) in the presence of 1 μM ryanodine (a) or 10 mM caffeine (b) recorded for 1 h. Ryanodine/caffeine was present for 10 min prior to glutamate plus glycine exposure, as well as throughout the entire experiment. Traces are representative of three independent experiments.

Ryanodine and caffeine but not thapsigargin inhibit DCD

Neurones were incubated with ryanodine (1 μM) or caffeine (10 mM) for 10 min prior to exposure to glutamate; the compounds remained present in the perfusate for the entire duration of the experiments (Fig. 5). Exposure to glutamate caused an abrupt elevation in $[Ca^{2+}]_i$ level (Figs 5a and b, 100 s) followed by a return to a newly established plateau. Activation of RyR resulted in a significant decrease in the percentage of the number of cells undergoing DCD (Table 1). Application of Tg did not increase mean onset time or incidence of DCD (Table 1). Further information concerning the pharmacological profile of the mechanism underlying both events was obtained by 2-APB and La^{3+}.

2-APB and La^{3+} modulate basal Ca^{2+} entry

Neurones were perfused with Ca^{2+}-free medium for 8–10 min, followed by re-introduction of the cation to the perfusing medium. 2-APB (100 μM) or $LaCl_3$ (10 μM) was present for the entire duration of the experiment (Fig. 6). It is evident that 2-APB diminished the peak Ca^{2+} influx by about 50%, while La^{3+} did not affect peak amplitude but caused an accelerated return towards baseline $[Ca^{2+}]_i$. An inhibition of basal calcium entry by 2-APB in platelets was previously reported (Dobrydneva and Blackmore 2001).

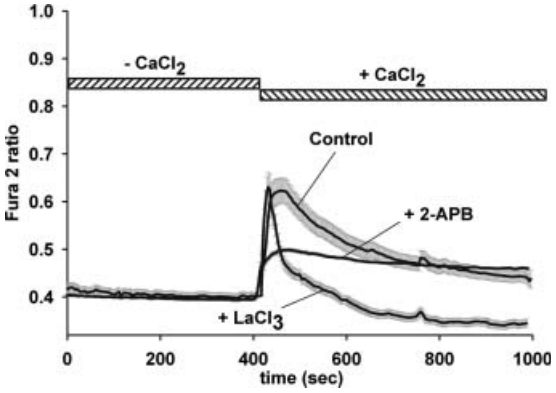

Fig. 6 Fura-2 ratio fluorescence of $[Ca^{2+}]_i$ of cortical neurones on basal Ca^{2+} entry. Cortical neurones were incubated in Ca^{2+}-free medium for 8–10 min (including the initial 400 s recording interval). $CaCl_2$ (2.6 mM) was re-introduced to the perfusing medium at 400 s. All curves were recorded in the absence of extracellular $MgCl_2$. 2-APB (100 μM) or $LaCl_3$ (10 μM) were present for the entire duration of the experiments. $t_{1/2}$: + $LaCl_3$: 78 ± 16*, + 2-APB: > 600 s*. Trace is representative of three independent experiments. One-way ANOVA, Tukey's post hoc analysis; *significant, compared to Fig. 3(c) –$MgCl_2$, $p < 0.05$.

Discussion

In the present study we demonstrated that the delayed Ca^{2+} deregulation induced by glutamate is diminished by 2-APB and La^{3+} in cultured cortical neurones. These agents are known to inhibit TRP and SOC channels. We did not find evidence for a thapsigargin or ryanodine-sensitive SOCE in these neurones. Furthermore, application of thapsigargin failed to aggravate mean onset time or the incidence of DCD (Table 1). In addition, activation of the RyR mitigated DCD.

Our results, excluding other obvious possibilities, could be consistent with the role of TRP channels operating in a manner independent of intracellular Ca^{2+} stores in inducing DCD. This notion is also supported by our observation that low concentration of 2-APB (30 μM) did not prevent DCD, arguing against the involvement of SOCE since at this concentration, the compound is a rapid and effective inhibitor of SOC channels (Dobrydneva and Blackmore 2001).

One of the proposed mechanisms of activation of SOC channels involves conformational coupling to the IP_3 receptor (Putney *et al*. 2001), and 2-APB was originally reported to block IP_3 receptors (Maruyama *et al*. 1997). However,

more recent studies have led to the consensus that the effect of this compound is due to a direct inhibition of SOC channels or proteins that mediate their activation (Dobrydneva and Blackmore 2001; Ma *et al*. 2002), rather than that of the IP_3 receptor itself (Bootman *et al*. 2002). SK&F 96365, an alternative SOC blocker (Merritt *et al*. 1990), did not prevent or delay DCD (Table 1).

The pharmacological promiscuity of 2-APB extends to many other targets, including the SERCA pump (at very high concentrations) (Bilmen *et al*. 2002), voltage-dependent K^+ channels (Wang *et al*. 2002), gap junctions (Harks *et al*. 2003) and the mitochondrial permeability transition pore (PTP) (Chinopoulos *et al*. 2003). Several lines of evidence prompted us to investigate the role of the PTP as a possible event upstream of the secondary Ca^{2+} rise: (i) application of the PTP inhibitor, cyclosporin A, or its non-immunosuppressant analogue, *N*-methyl-valine-4-cyclosporin, prevents the delayed Ca^{2+} rise in striatal (Alano *et al*. 2002) and hippocampal neurones (Vergun *et al*. 1999), but not in cerebellar granule cells (Castilho *et al*. 1998); (ii) 2-APB (and bongkrekic acid) inhibits high Ca^{2+}-induced PTP in isolated brain mitochondria (Chinopoulos *et al*. 2003); (iii) inhibition of the mitochondrial ANT with bongkrekic acid prevents NMDA receptor-mediated apoptosis of cerebrocortical neurones (Budd *et al*. 2000); (iv) a prerequisite for PTP induction is mitochondrial Ca^{2+} uptake (Zoratti and Szabo 1995) and DCD is eliminated when mitochondrial membrane potential is collapsed in the presence of the respiratory inhibitor, rotenone, plus the mitochondrial ATPase inhibitor, oligomycin (Nicholls and Budd 1998). Our results do not favour the involvement of PTP upstream of the occurrence of DCD, since neither cyclosporin A nor bongkrekic acid prevented or delayed DCD onset (Fig. 2, Table 1). Unfortunately, we were unable to obtain *N*-methyl-valine-4-cyclosporin, previously shown to inhibit PTP more effectively than cyclosporin A in mitochondria from nervous tissue (Khaspekov *et al*. 1999). In addition, brain mitochondria exhibit diminished sensitivity to inhibition of PTP by cyclosporin A in the presence of physiological concentrations of adenine nucleotides and Mg^{2+} but are responsive to bongkrekic acid (Chinopoulos *et al*. 2003).

In view of the fact that DCD is abolished by pharmacological inhibitors of TRP and SOC channels, the question arises as to whether DCD is a SOCE event. Relevant to this, it was previously reported that Ca^{2+} entering through either voltage- or receptor-operated channels provides a trigger to stimulate Ca^{2+} release from neuronal internal stores (Lipscombe *et al*. 1988). However, SOCE has not been demonstrated in every type of excitable cells originating from nervous tissue (Putney 2003). Our findings led us to conclude that cortical neurones do not exhibit authentic capacitative Ca^{2+} entry for the following reasons: (i) application of thapsigargin prior to and during re-introduction of $CaCl_2$ to the perfusing medium failed to potentiate the rise of fura-2 ratio compared with the absence of the SERCA inhibitor and, counter-intuitively, thapsigargin *decreased* Ca^{2+} influx (Fig. 3d). As a proof-of-protocol, astrocytes and endothelial cells subject to identical conditions exhibited robust CCE (not shown). However, neurones treated with thapsigargin in a Ca^{2+}-free medium in the absence or presence of EGTA exhibited reduction of $[Ca^{2+}]_{ER}$ and depletion of intracellular Ca^{2+} stores, respectively (Fig. 3a); (ii) neither ryanodine nor caffeine application prior to and during re-introduction of $CaCl_2$ to the perfusing medium led to SOCE, as compared with the absence of these agents (Fig. 4); (iii) cortical neurones exhibit 'basal Ca^{2+} entry' (Figs 3c and d) even when thapsigargin-sensitive intracellular stores are not emptied (Fig. 3a, top curve). This latter mode of Ca^{2+} entry merits further investigation.

Concerning the unexpected observations on the effects of ryanodine/caffeine on DCD, we cannot prove (or disprove) that in the presence of ryanodine there is a common mechanism underlying the amelioration of DCD and the moderate acceleration of decay of the $[Ca^{2+}]_i$ to the baseline seen in Fig. 4(a). Caffeine has been reported to inhibit the IP_3 receptor (Ehrlich *et al*. 1994). Indeed, it inhibited DHPG-induced $[Ca^{2+}]_i$ transients (not shown), which caused liberation of Ca^{2+} from intracellular stores mediated by the IP_3 receptor subsequent to activation of the mGluR1 (Nakamura *et al*. 2000) and therefore, a possible effect of caffeine on the IP_3R cannot be excluded. Although xestospongin C has been reported to inhibit the IP_3R (Gafni *et al*. 1997), it failed to abolish DCD or DHPG-induced $[Ca^{2+}]_i$ transients (10 μM xestospongin C, not shown). Inhibition of the SERCA pump by thapsigargin did not alter DCD incidence or mean onset time (Table 1). It is, however, possible that potentiation of DCD by thapsigargin was not detected under the conditions used in our experiments because over 85% of the cells underwent DCD in the absence of this modulator of intracellular Ca^{2+} stores.

Diverse pathological conditions of the central nervous system converge to excitotoxicity, a process characterized by excessive synaptic release of glutamate, which, in turn, activates post-synaptic glutamate receptors (Sattler and Tymianski 2001). As a consequence of the latter, severe neuronal Ca^{2+} and Na^+ loading occurs (Choi 1987), culminating in cell death (Arundine and Tymianski 2003). Events comparable with delayed calcium deregulation occur *in vivo* under pathological conditions (Silver and Erecinska 1992; Rothman and Olney 1995) and thus, investigation of DCD *in vitro* provides a model to study the events downstream to glutamate receptor activation. Although a profound increase in $[Ca^{2+}]_i$ unequivocally triggers early neurodegeneration (Tymianski *et al*. 1993c), the exact mechanism(s) responsible for the loss of $[Ca^{2+}]_i$ homeostasis remain obscure (Choi 1995). A large body of evidence (see introduction) supports the notion that the secondary rise is due to a Ca^{2+} influx pathway, supported by the discovery of a non-selective cation current appearing subsequent to NMDA application,

termed post-exposure current (I_{pe}) (Chen *et al*. 1997). In addition, it was recently demonstrated that anoxic neuronal death, which is linked to excitotoxicity (Goldberg *et al*. 1987; Goldberg and Choi 1993), is mediated through activation of TRPM7 (Aarts *et al*. 2003).

TRP channels emerge as obvious candidates for DCD on the basis of their intense expression in the central nervous system (Montell *et al*. 2002) and their high Ca^{2+} conductance (Zitt *et al*. 2002). Inexorably, the large number of TRP family members, together with their combinatorial co-assembling tendency and the lack of individually specific inhibitors, contributes to an inability to pinpoint the culprit channel in the present study. However, some of our results, together with the recent literature (Aarts *et al*. 2003), imply that DCD may be due to activation of TRPM7 (also known as LTRPC7/MIC/MagNuM) (Nadler *et al*. 2001; Clapham 2002). TRPM7 operates independently of store depletion (Prakriya and Lewis 2002b), is inhibited by 2-APB (Prakriya and Lewis 2002a) and La^{3+} (Runnels *et al*. 2001), is expressed in the mammalian brain (Runnels *et al*. 2001) and receives strong negative feedback by intracellular Mg^{2+} (Nadler *et al*. 2001). We could not achieve silencing of TRPM7 expression in cultured cortical neurones with RNA interference technology (tested by immunocytochemical detection on the cultures using a polyclonal antibody against TRPM7, not shown) based on the mouse sequence homologue (accession number: AY032951, GeneBank); unfortunately, the rat homologue sequence is not yet available.

The inability of La^{3+} to inhibit peak basal Ca^{2+} influx does not necessarily rule out the involvement of TRP channels in this effect, because at least two members of the TRP family (TRPC4 and 5) exhibit potentiation of their current by micromolar levels of La^{3+}, while other members are inhibited (Clapham *et al*. 2003). Given the complex combinatorial co-assembly of individual TRP channels within the same cell (Hofmann *et al*. 2002; Strubing *et al*. 2003), the effect of La^{3+} might reflect the combined result of two effects. In addition, the same TRP channel (hTRPC3) exhibits a cell-type-specific mode of activation and response to pharmacological inhibition (Trebak *et al*. 2002).

The potential identification of a novel molecular target amenable to pharmacological manipulation greatly expands the prospects for treating acute and chronic neurological disorders associated with excitotoxicity (Montell 2001; Wissenbach *et al*. 2004). This approach avoids excitatory neurotransmission blockade and therefore, lacks the serious drawbacks of glutamate receptor antagonists that contributed to the failure of clinical trials concerning stroke treatment (Ikonomidou and Turski 2002; Muir and Lees 2003). A more challenging field will be to understand why cortical neurones express Ca^{2+}-permeable, non-selective cation channels while they are so well equipped with VDCCs and ligand-gated cation receptors.

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