Mitochondria accumulate Ca^{2+} following intense glutamate stimulation of cultured rat forebrain neurones

R. James White and Ian J. Reynolds*

Center for Neuroscience and Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

1. In cultures of rat forebrain neurones, mitochondria buffer glutamate-induced, NMDA receptor-mediated Ca^{2+} influx. Here, we have used the fluorescent calcium indicator, indo-1 AM to record $[Ca^{2+}]_i$ from single cells. We varied either the glutamate concentration or the duration of exposure to investigate the cellular mechanisms recruited to buffer $[Ca^{2+}]_i$ within different stimulation protocols.

2. For a 15 s stimulus, the recovery time doubled as the glutamate concentration was raised from 3 to 300 μM. Changing the duration of exposure from 15 s to 5 min increased the recovery time tenfold even when the glutamate concentration was held at 3 μM.

3. We used a selective inhibitor of the mitochondrial Na^+–Ca^{2+} exchange, CGP-37157. When applied immediately after a 15 s, 100 μM glutamate challenge, CGP-37157 consistently caused a rapid fall in $[Ca^{2+}]_i$ followed by a slow rise after the drug was washed out. A similar pattern was seen with the 5 min, 3 μM glutamate stimulus. The effects of CGP-37157 are consistent with the release of substantial mitochondrial Ca^{2+} stores during recovery from an intense glutamate stimulus.

4. These studies suggest that mitochondria become progressively more important for buffering glutamate-induced Ca^{2+} loads as the stimulus intensity increases. The recovery of $[Ca^{2+}]_i$ to baseline following glutamate removal is critically regulated by the release of Ca^{2+} from mitochondrial stores via mitochondrial Na^+–Ca^{2+} exchange. The data highlight a previously under-appreciated role for $[Na^+]_i$ in the regulation of $[Ca^{2+}]_i$ in central neurones.

Ca^{2+} appears to play a critical role in the pathophysiology underlying necrotic cell death in a number of different tissues including neurones, cardiac myocytes and hepatocytes (Schanne, Kane, Young & Farber, 1979; Choi, 1995; Hess & Kukreja, 1995). In the central nervous system, Ca^{2+} is probably an important mediator of neuronal damage following ischaemia or trauma (Palmer, Marion, Botscheller, Swedlow, Styren & DeKosky, 1993; Choi, 1995). The term excitotoxicity has been coined to indicate the prominent role played by the excitatory neurotransmitter glutamate, in these types of brain injury (Michaels & Rothman, 1990). Stimuli which produce excitotoxic cell death in cultures of forebrain neurones trigger large initial transients in free intracellular calcium, $[Ca^{2+}]_i$, as well as a late, final rise in $[Ca^{2+}]_i$ (Randall & Thayer, 1992; Tymianski, Charlton, Carlen & Tator, 1993); however, measurements of $[Ca^{2+}]_i$ have not been helpful in predicting cell death (Hartley, Kurth, Bjerkness, Weiss & Choi, 1993; Tymianski *et al.* 1993). Instead, several investigators have proposed that the total amount of Ca^{2+} entering the cell during the brief excitotoxic stimulus (the Ca^{2+} 'load') is an important determinant of neuronal demise (Hartley *et al.* 1993; Eimerl & Schramm, 1994). A definition of how the Ca^{2+} load is buffered and identification of the Ca^{2+}-dependent effectors of cell death remain as two of the largest unsolved pieces in the excitotoxicity puzzle (Tymianski *et al.* 1993; Zhang, Dawson, Dawson & Snyder, 1994; Reynolds & Hastings, 1995; White & Reynolds, 1996).

A number of recent studies in cultures of central neurones have thus sought to identify the mechanisms that govern recovery following increases in $[Ca^{2+}]_i$ (Bleakman, Roback, Wainer, Miller & Harrison, 1993; White & Reynolds, 1995). The cell types and approaches have differed but an emerging consensus is that mitochondrial Ca^{2+} uptake plays a prominent role in the ability of the neurone to manage large $[Ca^{2+}]_i$ transients, particularly those induced by glutamate (Wang, Randall & Thayer, 1994; Kiedrowski & Costa, 1995; White & Reynolds, 1995). Mitochondria accumulate Ca^{2+} through a membrane potential-dependent Ca^{2+} uniporter, a protein complex inhibited by Ruthenium Red (Broekemeier, Krebsbach & Pfeiffer, 1994; Wang *et al.* 1994).

* To whom correspondence should be addressed at the Department of Pharmacology, E1354 Biomedical Science Tower, Pittsburgh, PA 15261, USA.

Once sequestered within the mitochondrion, Ca^{2+} has a number of routes available for egress, but none of them has been studied systematically in neurones. These mechanisms have, however, been studied in hepatocytes and in these non-excitable cells two different systems govern controlled Ca^{2+} efflux from mitochondria (Gunter, Gunter, Sheu & Gavin, 1994). The first is Na^+ independent and couples the hydrolysis of intramitochondrial pyridine nucleotides with mitochondrial Ca^{2+} efflux. The second and less significant pathway in hepatocytes is Na^+ dependent and involves the exchange of Na^+ for Ca^{2+}, although the stoichiometry of that reaction remains contested (Jung, Baysal & Brierley, 1995). Two other mechanisms for uncontrolled mitochondrial Ca^{2+} release have been described. When activated, the permeability transition pore allows the passage of all solutes under 2 kDa, but its role in physiological Ca^{2+} transport remains the subject of much debate (Bernardi, Broekemeier & Pfeiffer, 1994; Gunter *et al.* 1994). Another route for *efflux* with a poorly defined physiological role is the above-mentioned Ca^{2+} uniporter, which has been shown to operate in reverse when mitochondria are depolarized (Broekemeier *et al.* 1994; Gunter *et al.* 1994). To date the available data suggest that the Na^+-dependent pathway governs controlled Ca^{2+} efflux from neuronal mitochondria (Satrustegui & Richter, 1984; Kiedrowski & Costa, 1995; Wang & Thayer, 1996).

Our previous study of neuronal $[Ca^{2+}]_i$ regulation utilized brief exposure to low glutamate concentrations with the goal of describing Ca^{2+} buffering following stimuli that are not usually considered excitotoxic (White & Reynolds, 1995). These experiments demonstrated an overlapping capacity of mitochondria and plasma membrane Na^+–Ca^{2+} exchange to buffer glutamate-induced $[Ca^{2+}]_i$ transients. The present investigation was designed to explore changes in the mechanism of Ca^{2+} buffering as stimuli approached an intensity likely to prove injurious. In typical cultures of rat forebrain neurones, a 5 min stimulation with 100 μM glutamate kills a majority of the neurones when viability is measured 24 h later (Hartley *et al.* 1993; Zhang *et al.* 1994). Two important features of the stimulus have changed in the transition from non-excitotoxic to excitotoxic, namely, the duration of exposure and the concentration of glutamate. Thus, in this set of experiments, we sought to describe the impact of systematically changing the glutamate concentration or the duration of exposure while holding the other variable constant. We hypothesized that higher concentrations and longer exposures would result in significantly longer recovery times. In subsequent experiments, we manipulated the ability of the mitochondria to buffer Ca^{2+} following these more intense stimulation protocols. We first used the mitochondrial uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to disable mitochondrial Ca^{2+} uptake. We then used CGP-37157, a benzothiazepine relative of diltiazem which specifically inhibits the mitochondrial Na^+–Ca^{2+} exchange, which is the Na^+-dependent pathway for mitochondrial Ca^{2+} efflux (Cox, Conforti, Sperelakis & Matlib, 1993). When this pathway was blocked, we found that mitochondria could accumulate large stores of Ca^{2+} following more intense glutamate stimulation. These data, coupled with those from relevant control experiments using CGP-37157, strongly suggest that the Na^+-dependent pathway is the predominant route for mitochondrial Ca^{2+} efflux in central neurones and that mitochondrial Ca^{2+} release elevates $[Ca^{2+}]_i$ for some time following glutamate removal. Taken together, our experiments with FCCP and CGP-37157 demonstrate the importance of mitochondrial Ca^{2+} uptake in buffering intense glutamate stimuli and also highlight a previously under-appreciated role for Na^+ in buffering glutamate-induced $[Ca^{2+}]_i$ changes.

METHODS

Cell culture

In the present study, all procedures using animals were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Pregnant female rats were anaesthetized by ether inhalation until consciousness was lost. The anaesthetized females were killed by decapitation, the pups were removed and also killed by decapitation. Forebrains from embryonic day-17 Sprague–Dawley rat pups were dissociated and cultured as previously described (White & Reynolds, 1995). We selected pyramid-shaped neurones for $[Ca^{2+}]_i$ recordings 12–18 days later; excluding those neurones with a resting fluorescent emission ratio which indicated a $[Ca^{2+}]_i$ greater than 150 nM or with a 490 nm emission signal which was less than thirtyfold greater than background. Each type of experiment described in this paper was generally performed on cells from four or more different culture dates.

Calcium measurements

Neurones were loaded for 45–55 min at 37 °C in a solution of 5 μM indo-1 AM in Hepes-buffered salt solution (HBSS), containing (mM): NaCl, 137; KCl, 5; $MgSO_4$, 0·9; $CaCl_2$, 1·4; $NaHCO_3$, 10; Na_2HPO_4, 0·6; KH_2PO_4, 0·4; glucose, 5·6; Hepes, 20; with pH adjusted to 7·4 with NaOH) supplemented with 5 mg ml^{-1} bovine serum albumin (BSA) to enhance dye penetration into the cells. The dye was washed and the cells were re-incubated at 37 °C for an additional 20–30 min to allow for uniform cleavage of the acetoxymethyl ester moiety on the dye.

We added a computer-controlled shutter to the dual emission microfluorimetry apparatus which has been described previously (White & Reynolds, 1995). The customized data acquisition software was modified to drive a Uniblitz VA10 shutter (Vincent Associates, Princeton, NJ, USA) which opened briefly to illuminate the neurones for 50 ms while the photomultiplier tubes were sampled. The shutter allowed us to increase the intensity of excitation while still reducing the light-associated changes in the dynamic range of the dye (Randall & Thayer, 1992; White & Reynolds, 1995). One time point was collected each second.

A coverslip was used for one experiment prior to disposal. As indicated in the results, a complete experiment involved a 3 min collection of baseline $[Ca^{2+}]_i$; a 15 s, 3 μM glutamate as an internal control; a 15 min recovery period; and finally, a second glutamate stimulus which was immediately followed by perfusion with the solution indicated. All $[Ca^{2+}]_i$ recordings were made at room temperature (21–23 °C) and flow across the laminar chamber was held constant at 20 ml min^{-1}. All glutamate stimulations were performed in the presence of 1 μM glycine.

Calibration

In situ calibration of the dye in cortical neurones was performed as a modification of that previously described (White & Reynolds, 1995). At the same time that the dye was being loaded, neurones were energy depleted for 30 min in a glucose-free cytoplasmic buffer solution (CBS), containing (mM): KCl, 120; NaCl, 10; $NaHCO_3$, 10; Hepes, 10; $MgSO_4$, 0·05; and KH_2PO_4, 1; supplemented with indo-1 AM, 5 μM; BSA, 5 mg ml^{-1}; 2-deoxyglucose, 1 mM; and antimycin-A, 1 μM. The dye and cell poisons were washed with CBS and the cells were returned to the incubator for another 20 min. This procedure depleted ATP as well as the ionic gradients required for calcium pumping activity and though the cells looked swollen they appeared well-loaded with dye.

After measuring baseline $[Ca^{2+}]_i$, the chamber fluid was manually exchanged for CBS which contained 4Br-A23187, 20 μM; nigericin, 5 μM; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 750 nM; 2-deoxyglucose, 1 mM; antimycin-A, 1 μM; and EGTA, 1 mM. After a new equilibrium was established, the fluid was exchanged with CBS which contained the 4Br-A23187, nigericin, FCCP, antimycin, and 2-deoxyglucose as above but substituted with 2 mM $CaCl_2$ in place of EGTA and included 500 μM glutamate and 10 μM glycine. We modelled this approach of intracellular manipulation after Wahl, Lucherini & Gruenstein (1990), in an attempt to disrupt the buffering mechanisms of the cell and thus approximate dye saturation more closely. Antimycin and 2-deoxyglucose collectively insured that the ATP reserves of the cell were depleted, while nigericin is a mitochondrial poison which also equilibrated H^+ across the plasma membrane. FCCP directly abrogated any residual mitochondrial Ca^{2+} buffering capacity. Interestingly, glutamate and glycine further increased the dye saturation beyond that produced by 4Br-A23187. In the absence of these harsh conditions, our calibration R_{max} (the fluorescence ratio measured in the presence of saturating amounts of ion) was substantially smaller, thus yielding calculated peak $[Ca^{2+}]_i$ which were often in the range of 50–100 μM. The present strategy gave rise to consistently high values for R_{max} and correspondingly reasonable values for $[Ca^{2+}]_i$.

Calibration data were collected from six to eight different cells and the mean was used in the equation described by Grynkiewicz, Poenie & Tsien (1985); in our most recent calibration, β was 4·56; R_{min} was 0·75; R_{max} was 6·91, (where β is an affinity constant and R_{min} is the fluorescence ratio measured in the nominal absence of ion). The equipment was recalibrated every time the bulb was changed or the optical pathway was manipulated in any way, generally every 3 months; the constant values given above changed little from calibration to calibration. Background fluorescence was collected after each experiment and subtracted from the observed raw data prior to calcium value calculations.

Analysis and statistics

For most of the data, time to recovery was measured from the time that the glutamate-free HBSS reached the chamber until the time at which the neurones first reached a $[Ca^{2+}]_i$ value equal to twice the $[Ca^{2+}]_i$ prior to glutamate stimulation. This value is indicated by the open arrow in Figs 1, 2, 4, 5 and 6.

As with any fluorometric measurement of intracellular ion concentrations, the actual $[Ca^{2+}]_i$ values reported here are estimates. In these experiments we may be overestimating $[Ca^{2+}]_i$ largely because of the difficulty in saturating the dye during calibration. In our analysis, therefore, we have generally avoided comparisons based upon peak $[Ca^{2+}]_i$ values and focused instead on the $[Ca^{2+}]_i$ values as the cells approached baseline, since estimates of $[Ca^{2+}]_i$ using the dye are much more accurate within the 50–500 nM range. Thus, most of our critical measurements were made within the range where indo-1 reflects $[Ca^{2+}]_i$ values more accurately. Additionally, comparisons of peak values were made using data acquired under internally controlled conditions.

All summaries are reported as the means ± the standard error of the mean (S.E.M.) unless otherwise noted. For the main body of data, statistical significance was measured using a one-way ANOVA as calculated by InStat v2.0 (Graph Pad Software, San Diego, CA, USA). *Post hoc* analyses were performed with a Newman–Keuls test. Variability in our sample sizes precluded the use of multifactorial ANOVA where such use might have been appropriate in comparing experiments with and without extracellular Ca^{2+}. Instead, we used multiple *t* tests with a Bonferroni correction to compensate for repeated tests. Welch's *t* tests, in which estimates of population variance are calculated for each sample, were used when the homogeneity of variance assumption was questionable. Tukey box plots (Norman & Streiner, 1994) were generated by Axum, v4.0 (Trimetrix, Seattle, WA, USA). Here the median is drawn as a straight line across the box, the outer limits of the boxes encompass the central half of the data, and the whiskers are drawn to the data points closest to but not exceeding the median ± (1·5 × inter-quartile range). The inter- quartile range is the box length or the difference between the two values which encompass the central 50% of the data. If the data were normally distributed, the whiskers would encompass 95% of the data points. Outliers are represented by downward triangles and far outliers are shown as circles. Finally, small diamonds with connected error bars placed in the centre of the boxes reflect the means ± the S.E.M. in order to illustrate the difference between the means and the medians. These plots were the most informative way to illustrate a large body of data with significant heterogeneity.

Reagents

Nominally calcium-free solutions contained no added calcium and no chelating agents. Unless noted otherwise, nominally sodium-free solutions were made with 137 mM *N*-methyl-D-glucamine (NMG) as the sodium substitute with 5 mM $KHCO_3$ in place of 5 mM KCl; the pH was adjusted to 7·4 with concentrated HCl. $NaHCO_3$ was left out of Na^+-free solutions and the $KHCO_3$ was the sole source of bicarbonate anion. Stock 750 μM FCCP solutions were made in methanol giving a final solvent concentration of 0·1%. Nigericin was made as a 10 mM stock in ethanol. Indo-1 AM and 4Br-A23187 were obtained from Molecular Probes, aliquoted in anhydrous dimethylsulphoxide, and frozen. CGP-37157 was a generous gift from Ciba-Geigy Pharmaceuticals, Basel, Switzerland. It was stored as a dry powder at room temperature; 25 mM stocks were prepared in anhydrous DMSO, frozen (−20 °C) and used within 1 month. The final solvent concentration was 0·1%. Cell culture supplies were purchased from Gibco and unless otherwise noted, all other reagents were of the highest grade available from Sigma.

RESULTS

Recovery time is prolonged with increasing glutamate concentration

In this study we recorded from 279 neurones which had a mean baseline $[Ca^{2+}]_i$ of 43 ± 0·1 nM. Figure 1 illustrates the experimental protocol used in this series of experiments. Each trial was begun by establishing a baseline after which we applied a 15 s, 3 μM glutamate stimulus as an internal control. In Fig. 1*A–E*, we maintained the duration of the

second glutamate exposure constant at 15 s and varied the concentration between 3 and 300 μM. We reasoned that the recovery time would increase as a function of glutamate concentration. Following the application of each glutamate stimulus the cells were perfused with nominally Ca^{2+}-free buffer for the first 4 min to preclude continued Ca^{2+} influx. The time at which the neurone first reached twice its basal value after the experimental stimulus is shown as an open arrow. The Tukey box plot in Fig. 1*F* summarizes the mean recovery time as a function of the glutamate concentration and illustrates the substantial heterogeneity which we observed.

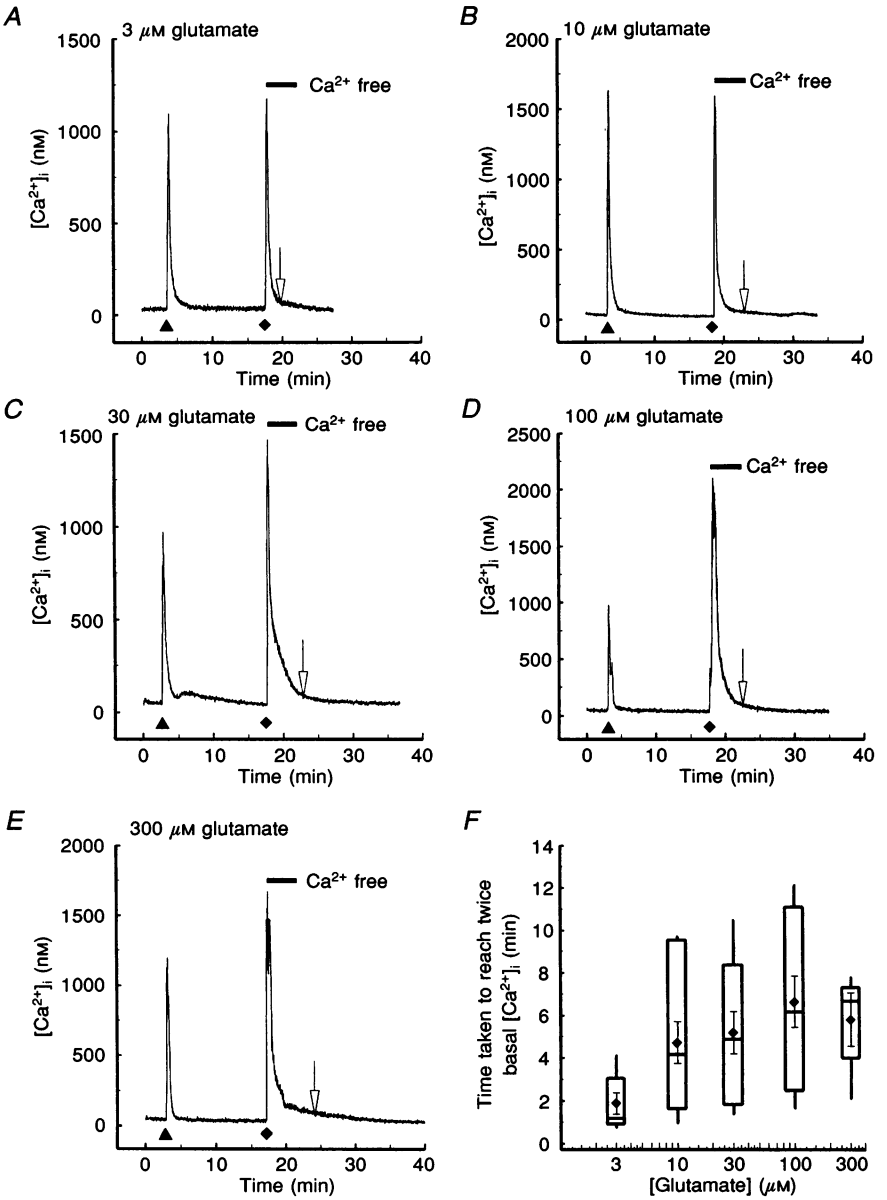

Figure 1. Increasing concentrations of glutamate slightly prolong the recovery time

A–E, cultured forebrain neurones were stimulated with a 15 s test dose of 3 μM glutamate and 1 μM glycine (▲). After a 15 min period of recovery, the neurones were again stimulated with a 15 s challenge of glutamate at the ◆; the concentration (from 3 to 300 μM) is noted in each panel. Immediately following the second glutamate challenge, the cells were bathed in Ca^{2+}-free media (filled bar) for 4 min to prevent influx after the stimulation period. The arrows denotes the time point at which the $[Ca^{2+}]_i$ first reached a value less than or equal to twice the baseline $[Ca^{2+}]_i$. Individual traces were chosen to reflect the mean of the recovery time data for the given set. The traces shown are thus reflective of a total of $n = 7$, 13, 13, 12, and 4 experiments, respectively, for *A–E*. *F* is a Tukey box plot (see Methods) illustrating the tremendous variability in the recovery time for this concentration–response curve; the box outlines the middle 50% of the data with the line across the centre reflecting the median. The means (◆) and S.E.M. (error bars) are shown.

Table 1. Extracellular Ca^{2+} decreases the recovery time when present in the wash solution following brief exposure to glutamate

	Ca^{2+} present during recovery			Ca^{2+} absent during recovery		
[Glutamate]	n	Peak $[Ca^{2+}]_i$ (μM)	Time to twice basal $[Ca^{2+}]_i$ (s)	n	Peak $[Ca^{2+}]_i$ (μM)	Time to twice basal $[Ca^{2+}]_i$ (s)
3 μM	13	1·07 ± 0·13	89 ± 16	7	1·64 ± 0·32	112 ± 30
10 μM	10	1·29 ± 0·12	124 ± 30	13	1·67 ± 0·28	284 ± 60
30 μM	13	1·63 ± 0·18	253 ± 58	13	1·86 ± 0·21	312 ± 60
100 μM	20	1·61 ± 0·14	172 ± 40	12	2·03 ± 0·19	399 ± 72*
300 μM	9	1·57 ± 0·20	265 ± 56	4	1·56 ± 0·18	349 ± 75

Forebrain neurones were stimulated with glutamate as illustrated in Fig. 1. The solution with which glutamate application was terminated either contained 1·4 mM Ca^{2+} or was nominally Ca^{2+} free. The peak $[Ca^{2+}]_i$ was measured as the largest $[Ca^{2+}]_i$ value achieved during the 15 s application of glutamate. The time to twice basal $[Ca^{2+}]_i$ was measured from the second that glutamate was washed out of the chamber until the time point at which the neurones first reached a $[Ca^{2+}]_i$ value equal to twice the $[Ca^{2+}]_i$ prior to glutamate stimulation. This value is illustrated by the open arrow in Fig. 1. Statistical significance was assessed with five individual t tests comparing the time to twice basal $[Ca^{2+}]_i$ with and without extracellular Ca^{2+} for each of the glutamate concentrations; a Bonferroni correction was made and the α value was set at $P < 0·01$ (* statistically significant difference).

We have previously shown that Ca^{2+} influx may occur after termination of the glutamate stimulus (White & Reynolds, 1995). We repeated the experiments illustrated in Fig. 1 with Ca^{2+} present in the recovery solution to determine the effect of extracellular Ca^{2+} on the recovery process. The overall variability of the data is similar to that illustrated in Fig. 1*F*, but one difference was notable. The data show a non-significant trend towards *longer* recovery times in the absence of extracellular Ca^{2+} with one of the values reaching significance (see Table 1). Table 1 also lists the peak $[Ca^{2+}]_i$ values for the second (experimental) peak illustrated in Fig. 1*A–E* and for the comparable experiments with Ca^{2+} present following the glutamate stimulus (not illustrated). Interestingly, although we are reluctant to make quantitative comparisons about the peak $[Ca^{2+}]_i$ values (see Methods), there is another non-significant trend which relates higher peak $[Ca^{2+}]_i$ with the acute removal of extracellular Ca^{2+}. Further investigation will be required to evaluate the hypothesis that rapid removal of extracellular Ca^{2+} induces a sudden alteration in the buffering process.

Recovery time is prolonged with increasing duration of glutamate stimulation

Figure 2 illustrates a similar set of experiments designed to investigate the degree to which duration of glutamate exposure influences recovery time. While maintaining the glutamate concentration constant at 3 μM, we varied the length of stimulation between 15 s and 5 min in panels *A–E*. Again, we hypothesized a positive relationship between the length of glutamate exposure and the subsequent recovery time. The open arrow in each trace illustrates the time at which the cells reached twice their basal $[Ca^{2+}]_i$, and the Tukey box plot in Fig. 2*F* demonstrates the significant relationship between duration of exposure and recovery time. Since varying the [glutamate] had no significant effect on recovery time (Fig. 1*F*), the data in Fig. 2*F* suggest that prolonging glutamate stimulation has a larger impact on the recovery time than does increasing agonist concentration.

The data in Table 2 show the lack of influence of extracellular Ca^{2+} on the recovery time in this protocol. This second set of data suggests that during the first 4 min following longer periods of glutamate stimulation the presence of extracellular Ca^{2+} is without effect on the recovery time. This lack of effect stands in contrast to the trend toward increased recovery time seen with Ca^{2+} absent from the wash buffer for the 15 s stimulations described in Table 1.

For exposure of durations of less than 5 min, the peak changes in $[Ca^{2+}]_i$ following longer glutamate stimulations with 3 μM were generally the same as those for the 15 s stimulation (Table 2, Fig. 2). However, on occasion the $[Ca^{2+}]_i$ continued to rise during extended exposure to glutamate and the peak $[Ca^{2+}]_i$ was measured as the highest $[Ca^{2+}]_i$ occurring at any time during the glutamate stimulation. The trace in Fig. 2*C* shows an exceptional case in which a 2·5 min stimulation afforded a larger peak response. On the other hand, the 5 min stimulation frequently gave rise to peaks in excess of the 15 s internal control as summarized in Table 2.

CGP-37157 inhibits the mitochondrial Na^+–Ca^{2+} exchange in forebrain neurones

We next sought to clarify the pathway(s) by which mitochondrial Ca^{2+} efflux occurs. Figure 3*A* illustrates an experiment in which FCCP was used to release Ca^{2+} from mitochondria. FCCP blocks the accumulation of Ca^{2+} by

dissipating the mitochondrial membrane potential and hence unmasking the tonic activity of the mitochondrial Ca^{2+} efflux pathways (Thayer & Miller, 1990; but also see Broekemeier *et al*, 1994). Despite the small size of FCCP-induced transients, it is clear that even in the absence of extracellular Ca^{2+}, a 30 s application of FCCP caused a measurable, reliable release of mitochondrial Ca^{2+} (Fig. 3*A*, first and second filled triangles). Twenty minutes in Na^+-free buffer is an interval sufficient to reduce $[Na^+]_i$ to near-zero in these neurones (Stout, Li-Smerin, Johnson & Reynolds,

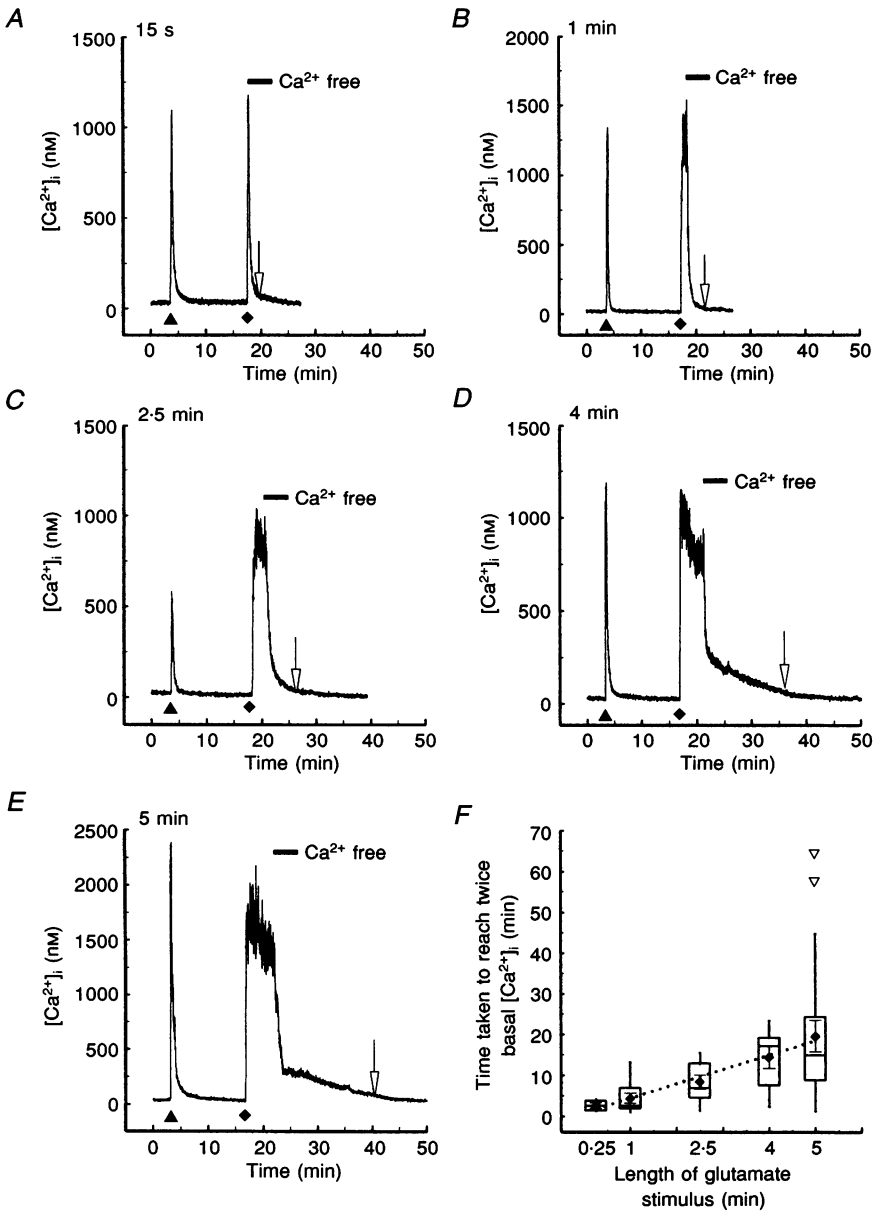

Figure 2. Increasing durations of exposure to 3 μM glutamate significantly prolong the recovery time

A–E, forebrain neurones were stimulated with a 15 s test dose of 3 μM glutamate and 1 μM glycine (▲); after a 15 min period of recovery, the neurones were again stimulated with a challenge of 3 μM glutamate beginning ◆; the duration of exposure (from 15 s to 5 min) is noted in each panel. Immediately following the second glutamate challenge, the cells were bathed in Ca^{2+}-free media (filled bar) for 4 min to prevent influx after the stimulation period. The arrow denotes the time point at which the $[Ca^{2+}]_i$ first reached a value less than or equal to twice the baseline $[Ca^{2+}]_i$. Individual traces were chosen to reflect the mean of the recovery time data for the given set. The traces shown are thus reflective of a total of $n = 7$, 11, 9, 8, and 20 experiments, respectively, for *A–E*. *F* is a Tukey box plot (see Methods) illustrating the correlation between duration of exposure and the recovery time. The box outlines the middle 50% of the data with the line across the centre reflecting the median. ▽ indicate outliers. Means (◆) and S.E.M. values (error bars) are shown. The best fit linear correlation of the mean data is illustrated as a dotted line (sum of squares = 201, d.f. = 3, $r^2 = 0{\cdot}979$, $F = 141$, $P = 0{\cdot}001$).

Table 2. Extracellular Ca^{2+} has no effect on recovery time when present in the wash solution following prolonged glutamate exposure

Length of glutamate exposure	Ca^{2+} present during recovery			Ca^{2+} absent during recovery		
	n	Peak $[Ca^{2+}]_i$ (μM)	Time to twice basal $[Ca^{2+}]_i$ (s)	n	Peak $[Ca^{2+}]_i$ (μM)	Time to twice basal $[Ca^{2+}]_i$ (s)
15 s	13	1·07 ± 0·13	89 ± 16	7	1·64 ± 0·32	112 ± 30
1 min	11	1·62 ± 0·19	235 ± 43	11	1·48 ± 0·09	267 ± 76
2·5 min	n.t.	—	—	9	1·25 ± 0·09	500 ± 99
4 min	n.t.	—	—	8	1·35 ± 0·13	868 ± 168
5 min	7	2·61 ± 0·95	1214 ± 326	20	2·21 ± 0·32	1179 ± 233

Forebrain neurones were stimulated with glutamate as illustrated in Fig. 2. The solution with which glutamate application was terminated either contained 1·4 mM Ca^{2+} or was nominally Ca^{2+} free. The peak $[Ca^{2+}]_i$ was measured as the largest $[Ca^{2+}]_i$ value achieved at any time during the exposure to glutamate. The time to twice basal $[Ca^{2+}]_i$ was measured from the second that glutamate was washed out of the chamber until the time point at which the neurones first reached a $[Ca^{2+}]_i$ value equal to twice the $[Ca^{2+}]_i$ prior to glutamate stimulation. This value is illustrated as the open arrow in Fig. 2. Statistical significance was assessed with three individual t tests comparing the time to twice basal $[Ca^{2+}]_i$ with and without extracellular Ca^{2+} for each of the exposure times. A Bonferroni correction was made and the α value was set at $P < 0{\cdot}016$. None of the differences were significant. n.t., not tested.

1996). The baseline $[Ca^{2+}]_i$ generally drifted up in the absence of extracellular Na^+ and there were occasionally unprovoked spikes in $[Ca^{2+}]_i$ (Fig. 3*A*, *). Once the cell was Na^+ depleted, FCCP was no longer capable of causing Ca^{2+} release from the mitochondria of forebrain neurones (Fig. 3*A*, third filled triangle) suggesting that the Ca^{2+} release under these conditions might be mediated by mitochondrial Na^+–Ca^{2+} exchange. We used a brief glutamate stimulus between FCCP challenges to prevent the mitochondria from becoming Ca^{2+} depleted (Fig. 3*A*, open triangles).

The experiment in Fig. 3*B* also shows the release of a small amount of mitochondrial Ca^{2+} after a 30 s application of FCCP in a Ca^{2+}-free buffer (Fig. 3*B*, first and second filled triangles). As before, we applied brief glutamate stimuli (Fig. 3*B*, open triangles) in between the FCCP challenges to prevent depletion of mitochondrial Ca^{2+} stores. Thirty seconds prior to the third FCCP trial, the mitochondrial Na^+–Ca^{2+} exchange inhibitor CGP-37157 was applied to the chamber. The absence of an FCCP-induced increase in $[Ca^{2+}]_i$ more strongly suggests that the elevation was due to mitochondrial Ca^{2+} efflux mediated by the mitochondrial Na^+–Ca^{2+} exchange. As illustrated, the drug works quite quickly and was rapidly and completely reversible.

Mitochondria buffer Ca^{2+} transients resulting from intense glutamate receptor stimulation

We next sought to evaluate the role of mitochondria in buffering the Ca^{2+} loads which resulted from these more intense glutamate challenges. In these experiments, the second glutamate stimulus was terminated by perfusing the chamber with the appropriate drug for a 2 min period. Utilizing FCCP to block mitochondrial Ca^{2+} uptake, Na^+-free buffer to reduce $[Na^+]_i$ as well as block plasma membrane Na^+–Ca^{2+} exchange and CGP-37157 to cause mitochondrial Ca^{2+} retention, we manipulated the ability of the neurones to buffer the Ca^{2+} loads resulting from these more intense glutamate stimuli. As with the controls, the cells were always perfused with a nominally Ca^{2+}-free buffer during the first 4 min following glutamate stimulation to eliminate the possibility of continued Ca^{2+} influx. Figure 4 shows the effect of manipulating the recovery processes following a brief exposure to a high glutamate concentration, while Fig. 5 shows parallel experiments following a prolonged stimulation with a low glutamate concentration.

FCCP routinely caused a robust increase in $[Ca^{2+}]_i$ when applied immediately after a 15 s, 100 μM glutamate stimulus (Fig. 4*B*); in similar experiments (7/13), the $[Ca^{2+}]_i$ increased beyond the value achieved during glutamate application to a mean peak of 3·46 ± 0·57 μM. Increases following termination of glutamate stimulation were never seen under control conditions. FCCP causes Ca^{2+} influx if extracellular Ca^{2+} is present (White & Reynolds, 1995). However, since we have eliminated extracellular Ca^{2+} from the recovery solution in the current protocol, the increase in $[Ca^{2+}]_i$ can only be ascribed to release of intramitochondrial stores. Although we occasionally found that FCCP application following a brief 100 μM stimulus dramatically prolonged the recovery time, there was a large degree of variability between neurones. The overall difference failed to reach statistical significance (Fig. 4*E*).

Application of a Na^+–Ca^{2+}-free buffer for 2 min following the 15 s, 100 μM stimulation is shown in Fig. 4*C*. Surprisingly, in these experiments the trend was to a lower, not higher, recovery time, although the data were scattered over a large range. Removal of extracellular Na^+ will eliminate the gradient for Ca^{2+} extrusion via the plasma membrane Na^+–Ca^{2+} exchanger. However, it will also reduce $[Na^+]_i$, thus decreasing the gradient forcing Na^+-dependent Ca^{2+} efflux from the mitochondria. Elimination of the extrusion mechanism would obviously impair $[Ca^{2+}]_i$ buffering if the neurones were dependent on the plasma membrane Na^+–Ca^{2+} exchange. On the other hand, reduction of $[Na^+]_i$ would slow efflux of Ca^{2+} from the mitochondria; the resultant effect of this latter manipulation on $[Ca^{2+}]_i$ recovery depends on the magnitude of the $[Na^+]_i$ decrease (Wang & Thayer, 1996).

Figure 4*D* illustrates the pattern seen in nine separate experiments in which a 15 s, 100 μM glutamate stimulation was terminated with 2 min of 25 μM CGP-37157. $[Ca^{2+}]_i$ dropped precipitously in the presence of CGP-37157 and in eight of nine experiments, $[Ca^{2+}]_i$ reached twice baseline in less than 4 min. CGP-37157 application was followed by a long wash in nominally Ca^{2+}-free buffer and despite the

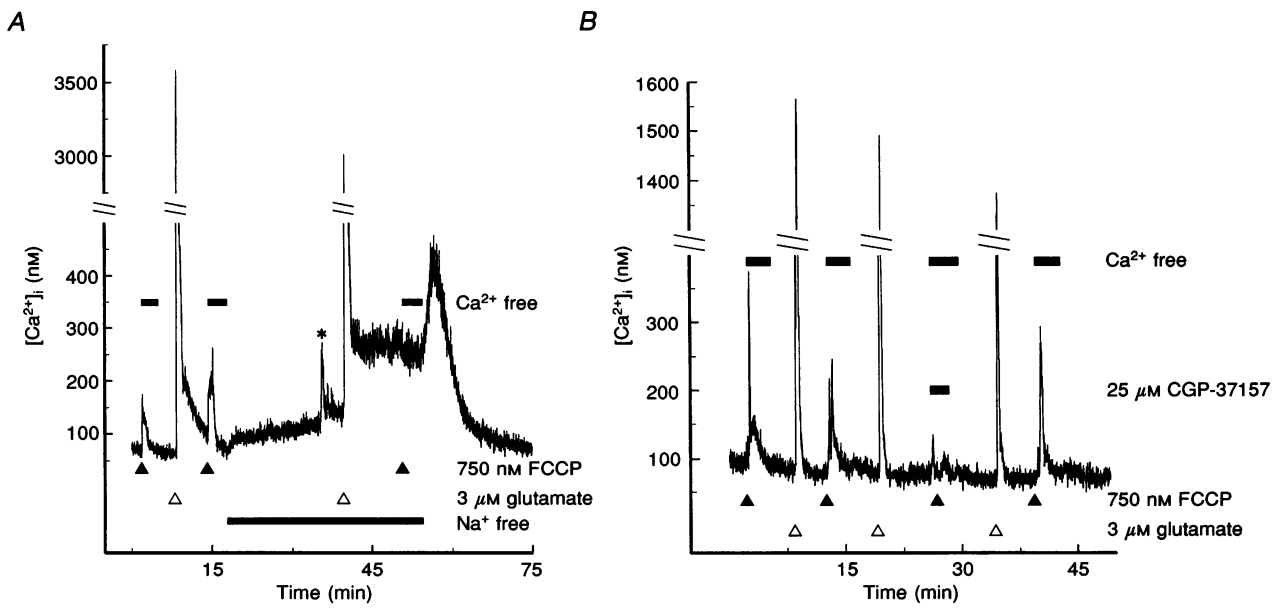

Figure 3. Mitochondrial Ca^{2+} efflux in forebrain neurones is Na^+ dependent and blocked by CGP-37157
After a brief collection of baseline data, this forebrain neurone (*A*) was challenged for 30 s with 750 nM FCCP applied in a Ca^{2+}-free buffer (▲); the stimulus gave rise to a small but measurable increase in $[Ca^{2+}]_i$, presumably by allowing mitochondrial Ca^{2+} efflux to exceed mitochondrial Ca^{2+} uptake. The duration of time spent in Ca^{2+}-free buffer is noted by the upper filled bars on the trace. The 15 s, 3 μM glutamate stimuli (△) insured that the mitochondria did not become Ca^{2+} depleted during the course of an experiment. The second, identical FCCP stimulus (second ▲) illustrates the qualitative reproducibility of the effect. After a brief period of recovery, the Na^+ was replaced with equimolar *N*-methyl-D-glucamine (lower filled bar) for a period sufficient to reduce intracellular Na^+ to near-zero levels. * denotes an unprovoked rise in $[Ca^{2+}]_i$ occasionally seen during the course of these harsh experimental conditions. A Ca^{2+}-containing, Na^+-free glutamate challenge (second △) again insured that mitochondria retained some releasable Ca^{2+}. A third FCCP stimulus was delivered (third ▲) without any perturbation in $[Ca^{2+}]_i$; the experimental manipulation for this third stimulus was in the absence of intracellular Na^+. Return of Na^+ and Ca^{2+} to the buffer resulted in a large and prolonged $[Ca^{2+}]_i$ rise before baseline levels were attained. This trace is illustrative of a total of five similar experiments in which the $[Ca^{2+}]_i$ did not rise if FCCP was applied when intracellular Na^+ was near-zero. After a brief collection of baseline data, this forebrain neurone (*B*) was also challenged for 30 s with 750 nM FCCP applied in a Ca^{2+}-free buffer (▲). The duration of time spent in Ca^{2+}-free buffer is noted by the filled bars at the top of the trace. The 15 s, 3 μM glutamate stimuli (△) insured that the mitochondria did not become Ca^{2+} depleted during the course of an experiment. The second, identical FCCP stimulus (second ▲) illustrates the qualitative reproducibility of the effect. Thirty seconds prior to application of the third FCCP challenge (third ▲), we added the mitochondrial Na^+–Ca^{2+}-exchange inhibitor CGP-37157 (25 μM, mid-level filled bar. CGP-37157 was present throughout the FCCP application and for 2 min thereafter). The increase in $[Ca^{2+}]_i$ which follows FCCP in the other three instances is not reproduced in the presence of CGP-37157. This trace also illustrates that CGP-37157 was entirely reversible and a final FCCP challenge (fourth ▲) elicits an $[Ca^{2+}]_i$ response quite similar to the first. This trace is illustrative of a total of four similar experiments in which the $[Ca^{2+}]_i$ response to FCCP was almost completely blocked by co-application of CGP-37157.

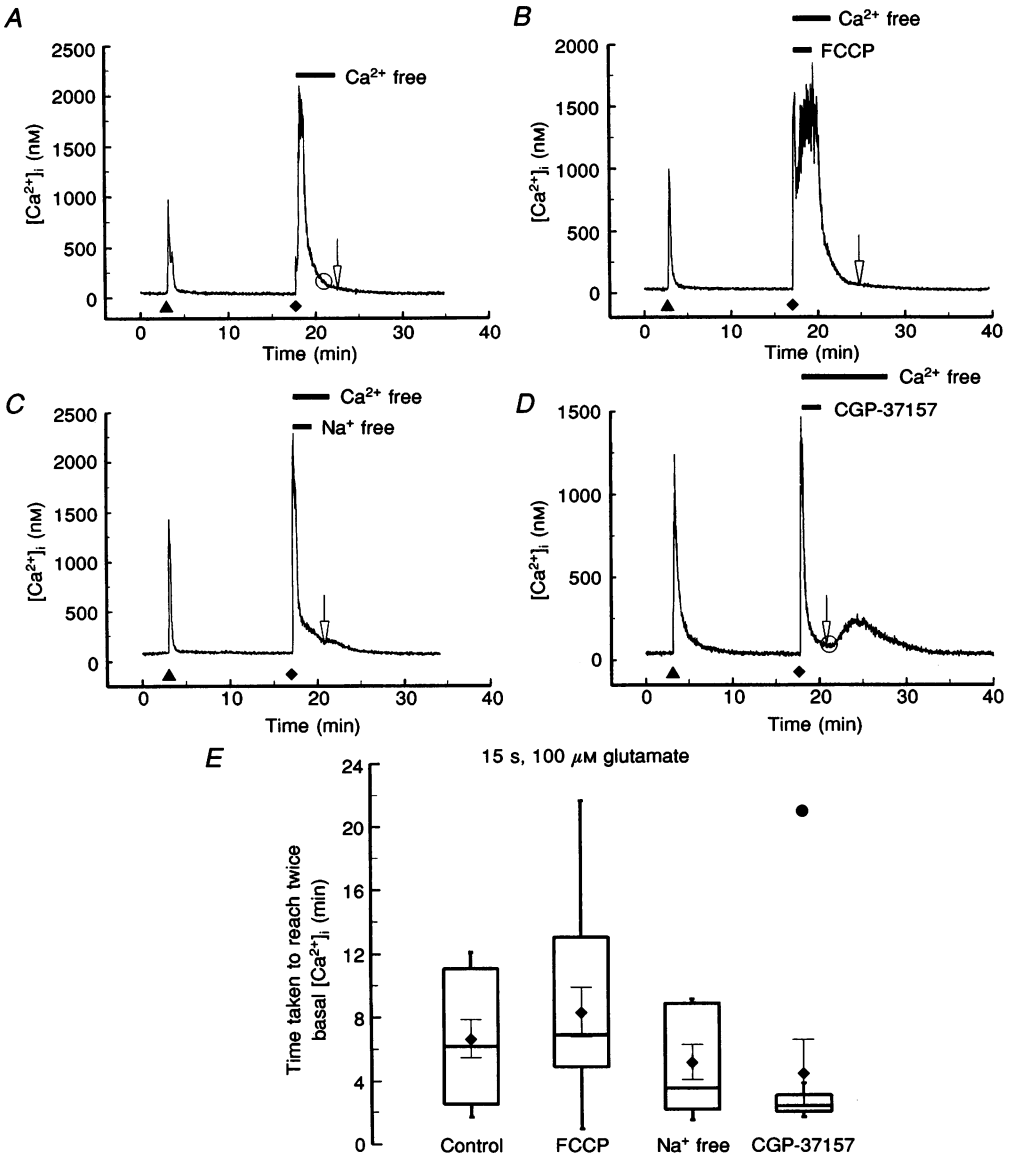

Figure 4. The role of mitochondrial Ca^{2+} buffering following brief exposure to high concentrations of glutamate

A–D, forebrain neurones were stimulated with 3 μM glutamate and 1 μM glycine for 15 s (▲). After a 15 min period of recovery, the neurones were again stimulated with a challenge of 15 s, 100 μM glutamate beginning ◆. The glutamate was immediately washed out with a Ca^{2+}-free buffer (upper filled bar) containing: buffer alone, *A*; 750 nm FCCP, *B*; NMDG-substituted for Na^+, *C*; 25 μM CGP-37157, *D*. Each of the test solutions was applied for a 2 min period (lower filled bar) and then washed out for an additional 2 min with Ca^{2+}-free buffer except in *D*, where the Ca^{2+}-free buffer was maintained throughout the period in which $[Ca^{2+}]_i$ was elevated. In each of the panels, the arrow denotes the time point at which the $[Ca^{2+}]_i$ first reached a value less than or equal to twice the baseline $[Ca^{2+}]_i$. Individual traces were chosen to reflect the mean of the recovery time data for the given set; the traces shown are thus reflective of a total of $n = 12$, 13, 9 and 9 experiments, respectively, for *A–D*. The circle around the trace in *A* and *D* marks the $[Ca^{2+}]_i$ at exactly 3 min after glutamate had been removed from the chamber. In *D*, that time point corresponds with a time 1 min after CGP-37157 had been removed from the chamber. *E*, tukey box plot (see Methods) summarizing the series of experiments outlined in *A–D* and illustrating the variability in the recovery times when FCCP, Na^+-free buffer, or CGP-37157 was applied immediately following a 15 s, 100 μM glutamate stimulus ($n = 12$, 13, 9 and 9, respectively, for the four box plots). The box outlines the middle 50% of the data with the line across the centre reflecting the median; ● is a far outlier (2·9 standard deviations greater than the mean). The means (◆) and S.E.M. values (error bars) are shown.

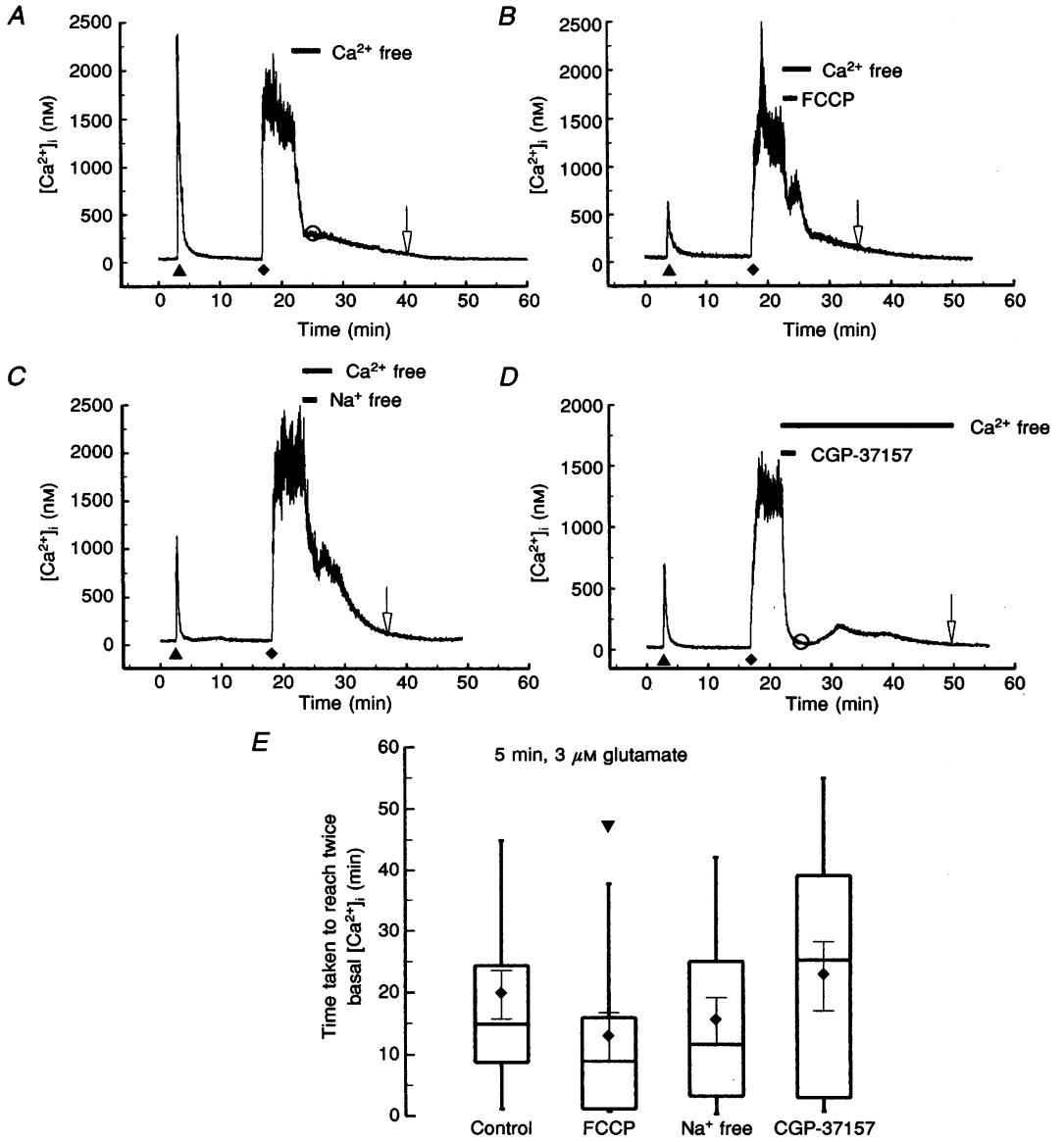

Figure 5. The role of mitochondrial Ca^{2+} buffering following prolonged exposure to low concentrations of glutamate

A–D, forebrain neurones were stimulated with 3 μM glutamate and 1 μM glycine for 15 s (▲). After a 15 min period of recovery, the neurones were again stimulated with a challenge of 5 min, 3 μM glutamate beginning at ◆. The glutamate was immediately washed out with a Ca^{2+}-free buffer (upper filled bar) containing: buffer alone, *A*; 750 nM FCCP, *B*; NMDG-substituted for Na^+, *C*; 25 μM CGP-37157, *D*. Each of the test solutions was applied for a 2 min period (lower filled bar) and then washed out for an additional 2 min with Ca^{2+}-free buffer. In *D* the Ca^{2+}-free buffer was maintained throughout the period in which $[Ca^{2+}]_i$ continued to rise to insure that we were measuring movement among intracellular compartments. In each of the panels, the arrow denotes the time point at which the $[Ca^{2+}]_i$ first reached a value less than or equal to twice the baseline $[Ca^{2+}]_i$. Individual traces were chosen to reflect the mean of the recovery time data for the given set; the traces shown are thus reflective of a total of $n = 20$, 13, 14 and 13 experiments, respectively, for *A–D*. The circle around the trace in *A* and *D* marks the $[Ca^{2+}]_i$ at exactly 3 min after glutamate had been removed from the chamber. In *D*, that time point corresponds with a time 1 min after CGP-37157 had been removed from the chamber. *E*, tukey box plot (see Methods) summarizing the series of experiments outlined in *A–D* and illustrating the variability in the recovery times when FCCP, Na^+-free buffer, or CGP-37157 was applied immediately following a 5 min, 3 μM glutamate stimulus ($n = 20$, 13, 14 and 13, respectively, for the four box plots). The box is as described in Fig. 4 but the ▼ is an outlier (2·3 standard deviations greater than the mean). The means (◆) and S.E.M. values (error bars) are shown.

prolonged absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ slowly rose after CGP-37157 was removed in all nine experiments. After attaining a mean peak height of 220 ± 60 nM, $[Ca^{2+}]_i$ steadily declined back to baseline over a 10–15 min period. Such a late rise in $[Ca^{2+}]_i$ rise was never seen in any of the control experiments.

The qualitative effect of CGP-37157 was striking and as graphed in Fig. 4*E*, the presence of CGP-37157 dramatically reduced the cell-to-cell variability seen in the control experiments as well as those modified by Na^+-free or FCCP-containing buffers (illustrated as the length of the Tukey box plot). However, the biphasic action of the drug made it difficult to quantify its effect with our standard method. In one of the nine experiments, the cell recovered rapidly but failed to reach twice basal $[Ca^{2+}]_i$ before the slow rise in $[Ca^{2+}]_i$ began. Our criteria for recovery time, therefore, reported a dramatically *longer* value for recovery than any of the control experiments despite the fact that the qualitative pattern exactly replicated the other eight experiments with CGP-37157. The outlier had a large effect on the overall mean ± S.E.M. thus preventing the difference from achieving statistical significance.

In the protocol illustrated in Figs 4*D* and 5*D*, drug washout was always followed by a slow rise and subsequent fall in $[Ca^{2+}]_i$. Thus, CGP-37157 had two opposite effects on the time to twice basal $[Ca^{2+}]_i$: the drug decreased it markedly for many of the neurones but increased it markedly for the remainder since the cells partially recovered, increased their $[Ca^{2+}]_i$ transiently and then recovered completely. We, therefore, chose to measure $[Ca^{2+}]_i$ 1 min after CGP-37157 was removed, the time when the effects of the drug were most striking (open circle, Figs 4*D* and 5*D*). We then compared that value to $[Ca^{2+}]_i$ measurements taken at the equivalent time point in control experiments. CGP-37157 reduced the between-cell variability with respect to the pattern of recovery and the time course of the recovery was almost identical from cell to cell. Thus, we were able to take the mean of the nine responses by comparing the $[Ca^{2+}]_i$ 1 min after CGP-37157 washout to the $[Ca^{2+}]_i$ achieved at the equivalent time in twelve control experiments (the open circle around the traces in Fig. 4*A* and *D*). $[Ca^{2+}]_i$ 1 min after CGP-37157 was removed from the chamber (i.e. 3 min after glutamate was washed out) was significantly different from that in control experiments (control: $[Ca^{2+}]_i$, 170 ± 40 nM, $n = 12$; CGP-37157: $[Ca^{2+}]_i$, 60 ± 10 nM, $n = 9$; Welch's $t = 2{\cdot}668$, d.f. = 12, $P = 0{\cdot}02$, where n is the number of cells used).

In a similar set of experiments, we manipulated the effectors of neuronal Ca^{2+} homeostasis following a 5 min, 3 μM glutamate challenge (Fig. 5). The first notable difference between the two basic glutamate protocols of Figs 4 and 5 is apparent in the control experiments illustrated in Figs 4*A* and 5*A*. The relatively complete and continuous recovery in Fig. 4*A* stands in contrast to the rapid initial recovery followed by a prolonged 'shoulder' seen in Fig. 5*A*. The response to mitochondrial uncoupling was also distinctive. Application of FCCP following the prolonged glutamate challenge (Fig. 5*B*) generally did not result in the robust increases in $[Ca^{2+}]_i$ seen when FCCP was used to terminate the brief 100 μM stimulus. The small amount of Ca^{2+} 'release' shown in Fig. 5*B* occurred in 3/14 experiments while a $[Ca^{2+}]_i$ increase which exceeded the glutamate-induced peak was also seen in an additional 3/14. However, most of the experiments showed no discernible rise in $[Ca^{2+}]_i$. When FCCP followed the brief 100 μM stimulus, the overall variability in the recovery time increased dramatically and many of the cells had recovery times longer than any of the controls (Fig. 4*E*). In contrast, when FCCP followed the longer 3 μM glutamate challenge, the variability was not markedly changed and the box plot was simply shifted to shorter recovery times with a 7 min change in the median (Fig. 5*B*). The single outlier prevented the mean from changing to the same extent as the median.

Removal of Na^+ from the extracellular buffer following the longer 3 μM glutamate stimulation produced no dramatic effect on the overall recovery time but did change the shape of the recovery in 6/14 experiments as illustrated in Fig. 5*C*. In these six experiments, return of Na^+ into the superfusate was heralded by a short-lived 'bump' in the $[Ca^{2+}]_i$. There was a mixture of responses to the Na^+-free manipulation as illustrated in Fig. 5*E*. However, like that seen with the brief 100 μM glutamate challenge, if removing extracellular Na^+ from the recovery solution had any overall effect, it was to *decrease* the time taken to reach twice basal $[Ca^{2+}]_i$.

Termination of the 5 min, 3 μM glutamate stimulus with a 2 min application of CGP-37157 resulted in a precipitous drop in $[Ca^{2+}]_i$ and again, $[Ca^{2+}]_i$ rose after the drug had been washed out despite the prolonged absence of extracellular Ca^{2+}. In this protocol many of the neurones did not recover to twice basal $[Ca^{2+}]_i$ immediately after CGP-37157 application. For example in Fig. 5*D*, the neurone did not arrive at twice basal $[Ca^{2+}]_i$ until almost 25 min after CGP-37157 had been washed out (open arrow). However, 1 min following the removal of CGP-37157 $[Ca^{2+}]_i$ once again proved to be a successful quantitative discriminator between the control and CGP-37157 modified recovery patterns (control: $[Ca^{2+}]_i$, 484 ± 159 nM, $n = 20$; CGP-37157: $[Ca^{2+}]_i$, 152 ± 41 nM, $n = 13$; Welch's $t = 2{\cdot}061$, d.f. = 21, $P = 0{\cdot}05$).

The rise in $[Ca^{2+}]_i$ following CGP-37157 washout specifically reflects mitochondrial Ca^{2+} accumulation

To test directly the proposition that CGP-37157 promotes the accumulation of Ca^{2+} by mitochondria following intense glutamate stimulation, we performed two sets of control experiments. In the first series, we utilized a protocol similar to that of Figs 4*D* and 5*D*. After the test stimulus, we applied a second 15 s, 3 μM glutamate challenge immediately followed by 2 min of CGP-37157 in a Ca^{2+}-free media (Fig. 6*A*). We hypothesized that the smaller amount of Ca^{2+} influx generated by the 15 s, 3 μM glutamate challenge would not cause sufficient mitochondrial Ca^{2+}

accumulation to result in the post-CGP-37157 $[Ca^{2+}]_i$ 'bump' seen in Figs 4*D* and 5*D*. It is clear that the marked and prolonged rise in $[Ca^{2+}]_i$ usually seen after CGP-37157 washout cannot be identified in Fig. 6*A*. This trace is representative of eleven similar experiments. There was also no difference from control (Fig. 1*A*) as assessed by the time to twice basal $[Ca^{2+}]_i$; (control, 112 ± 30 *vs.* CGP-37157, 86 ± 14 s). However, the $[Ca^{2+}]_i$ value 3 min after glutamate washout did indicate a significant difference between controls and the CGP-37157 treated cells (control: $[Ca^{2+}]_i$, 63 ± 7 nM, $n = 7$; CGP-37157: $[Ca^{2+}]_i$, 40 ± 5 nM, $n = 11$; Welch's $t = 2{\cdot}674$, d.f. $= 11$, $P = 0{\cdot}022$). Thus, the efflux of mitochondrial Ca^{2+} prolongs recovery after even modest glutamate receptor activation. Yet the brief 3 μM glutamate stimulus apparently did not cause sufficient accumulation of mitochondrial Ca^{2+} stores to generate the characteristic elevation of $[Ca^{2+}]_i$ after washout of CGP-37157.

In a second set of experiments, we demonstrated that the slow rise in $[Ca^{2+}]_i$ following CGP-37157 washout was dependent upon mitochondrial Ca^{2+} uptake. We hypothesized that if we blocked mitochondrial Ca^{2+} uptake following an intense stimulation then we would no longer be able to appreciate the rise in $[Ca^{2+}]_i$ following CGP-37157 application and washout. Thus, we applied CGP-37157 and FCCP together for 2 min immediately following a 15 s, 100 μM glutamate stimulus (Fig. 6*B*). The trace illustrates the apparent 'release' of Ca^{2+} from mitochondria which is also shown in Fig. 4*B*; 'release' was seen in approximately the same fraction of cells as in the analogous experiments illustrated in Fig. 4*B* (3/6). $[Ca^{2+}]_i$ recovered after a prolonged period of elevation and the latter portion of the trace demonstrates the critical finding. The slow, prolonged rise usually present after CGP-37157 application was not seen if the cells were simultaneously treated with FCCP; in the absence of FCCP, the 15 s, 100 μM stimulus uniformly resulted in a late $[Ca^{2+}]_i$ rise. Thus, mitochondrial uptake of Ca^{2+} was required if we were to identify an increase in $[Ca^{2+}]_i$ following application and removal of CGP-37157.

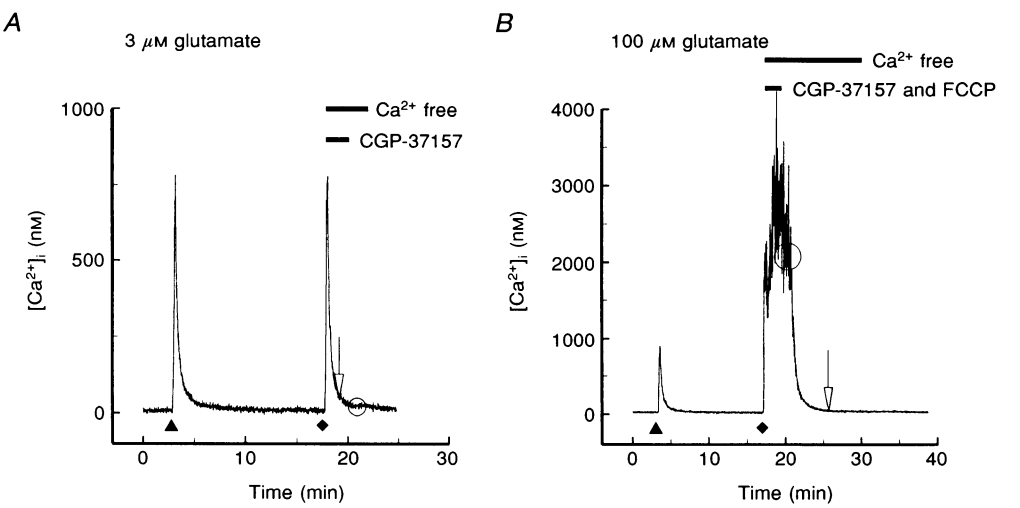

Figure 6. Increases in $[Ca^{2+}]_i$ following washout of CGP-37157 reflect accumulation of mitochondrial Ca^{2+} stores

A, this forebrain neurone was stimulated with 3 μM glutamate and 1 μM glycine for 15 s (▲). After a 15 min period of recovery, the cell was again stimulated with a challenge of 15 s, 3 μM glutamate beginning at ◆. The glutamate was immediately washed out with a 2 min bath of 25 μM CGP-37157 in Ca^{2+}-free buffer. The CGP-37157 treatment was terminated with another 2 min wash in Ca^{2+}-free buffer and no increase in $[Ca^{2+}]_i$ was observed. The arrow denotes the time point at which the $[Ca^{2+}]_i$ first reached a value less than or equal to twice the baseline $[Ca^{2+}]$i. This trace was chosen to reflect the mean of the recovery time data for 11 total experiments. The ○ around the trace marks the $[Ca^{2+}]_i$ at exactly 3 min after glutamate had been removed from the chamber; that time point corresponds with the time 1 min after CGP-37157 had been removed from the chamber. *B*, this forebrain neurone was stimulated with 3 μM glutamate and 1 μM glycine for 15 s (▲). After a 15 min period of recovery, the cell was challenged with 15 s, 100 μM glutamate beginning at ◆. The glutamate was immediately washed out with a 2 min bath of 25 μM CGP-37157 and 750 nM FCCP in Ca^{2+}-free buffer. This combined treatment was terminated with a prolonged wash in Ca^{2+}-free buffer and no increase in $[Ca^{2+}]_i$ was observed. The arrow denotes the time point at which the $[Ca^{2+}]_i$ first reached a value less than or equal to twice the baseline $[Ca^{2+}]_i$. This trace was chosen to reflect the mean of the recovery time data for a total of 6 experiments. The circle around the trace marks the $[Ca^{2+}]_i$ at exactly 3 min after glutamate had been removed from the chamber. That time point corresponds to the time 1 min after CGP-37157 had been removed from the chamber.

DISCUSSION

In this series of experiments, we have investigated the role which mitochondria play in governing the recovery of $[Ca^{2+}]_i$ following glutamate stimulation. By rapidly and reversibly blocking the major Ca^{2+} efflux pathway, the mitochondrial Na^+–Ca^{2+} exchange inhibitor CGP-37157 allowed us to demonstrate that intense glutamate stimulation causes mitochondria to accumulate stores of Ca^{2+}. High concentrations of glutamate were effective in loading mitochondria with Ca^{2+}, even when applied for as little as 15 s. In contrast, a brief 3 μM glutamate stimulus did not cause measurable mitochondrial Ca^{2+} storage (Fig. 6*A*). This lack of accumulation is interesting since our earlier work clearly illustrates the important contribution that mitochondria make in buffering $[Ca^{2+}]_i$ back to baseline following the 15 s, 3 μM stimulus (White & Reynolds, 1995). Thus, accumulation of mitochondrial Ca^{2+} seems to be specifically associated with more intense glutamate stimulation.

Mitochondria play a critical role in shaping the $[Ca^{2+}]_i$ response to glutamate-induced Ca^{2+} loads

A number of different investigations suggest that mitochondria buffer glutamate-induced Ca^{2+} loads in central neurones (Wang *et al.* 1994; Kiedrowski & Costa, 1995; White & Reynolds, 1995, 1996; Wang & Thayer, 1996; but see Budd & Nicholls, 1996). Since the mitochondria have an impressive capacity to buffer Ca^{2+}, we hypothesized that the larger Ca^{2+} load induced by intense glutamate stimulation would increase the relative importance of mitochondrial Ca^{2+} uptake. In the current set of experiments, we intensified the glutamate stimulus by increasing the glutamate concentration or by utilizing a longer exposure to a fixed low concentration. Interestingly, the two protocols resulted in differential loading of mitochondrial stores. A 15 s challenge with 100 μM glutamate followed by the protonophore FCCP produced an immediate increase in $[Ca^{2+}]_i$ in over half of the experiments (Figs 4*B* and 6*B*). This pattern was quite different from that seen when FCCP was applied immediately following a 15 s, 3 μM glutamate challenge (White & Reynolds, 1995). In those experiments, Ca^{2+} was present in the recovery solution and even with continued Ca^{2+} influx, $[Ca^{2+}]_i$ never rose higher than that achieved during glutamate application. Even a 5 min challenge with 3 μM glutamate followed by FCCP was generally ineffective at producing a marked rise in $[Ca^{2+}]_i$ (Fig. 5*B*). In fact, under these latter conditions, FCCP treatment caused a non-significant trend towards decreased length of recovery as compared with control (Fig. 5*B* and *E*).

It is possible to reconcile these disparate findings. Usually we did not see a significant change in the recovery time as a 15 s stimulation was modified with an increased concentration of 3–100 μM glutamate (Fig. 1). However, FCCP was able to differentiate the degree to which mitochondria were 'loaded' with Ca^{2+}. In non-excitable cells, mitochondria appear to be critically positioned as buffers for microdomains of large Ca^{2+} transients (Rizzuto, Brini, Murgia & Pozzan, 1993). It may be that neuronal mitochondria are similarly positioned to buffer large Ca^{2+} loads induced by intense glutamate stimulation.

In the brief stimulation protocol, it seems reasonable to suggest that neurones with large absolute levels of Ca^{2+} influx can be identified by long recovery times and the presence of an FCCP-releasable pool. The overall variability under control conditions reflected in Fig. 1*F* suggests that some cells experienced much larger Ca^{2+} loads following a 100 μM glutamate stimulus than others. In this heterogenous preparation of forebrain neurones, variable expression of Ca^{2+}-permeable glutamate receptor subtypes and subclasses of voltage-gated Ca^{2+} channels would influence the absolute amount of Ca^{2+} influx. The initial metabolic state of the individual cells could also be another important source of the large between-cell differences. Further experiments will be necessary in order to differentiate between these and other possibilities which might have contributed to the scatter in the data which we observed.

Mitochondrial Ca^{2+} efflux prolongs glutamate-induced $[Ca^{2+}]_i$ transients

Several lines of evidence suggest that mitochondria can contribute to the extended elevation of $[Ca^{2+}]_i$ following intense stimulation in both peripheral and central neurones (Thayer & Miller, 1990; Kiedrowski & Costa, 1995; Wang & Thayer, 1996; White & Reynolds, 1996). Once accumulated during the period of greatest influx, the Ca^{2+} stored by mitochondria is slowly released at a rate dictated by the $[Na^+]_i$ as well as the ability of the plasma membrane to extrude Ca^{2+} from the cell. In the complete absence of mitochondrial Ca^{2+} efflux, $[Ca^{2+}]_i$ recovery actually proceeds faster (White & Reynolds, 1996) while the mitochondria retain large amounts of Ca^{2+}.

The extended exposure to a low glutamate concentration did result in a significantly protracted period of $[Ca^{2+}]_i$ recovery (Fig. 2*F*). Prolonged elevation of mitochondrial matrix Ca^{2+} results in the formation of Ca^{2+}–phosphate and Ca^{2+}–protein complexes which are not readily releasable (Nicholls, 1985; Gunter *et al.* 1994). Although the *in vivo* rate of formation is unknown, these two processes are likely to be relatively slow in comparison with the rate of uptake. In the 5 min protocol, the accumulation of mitochondrial Ca^{2+} would have occurred more slowly and the accumulated Ca^{2+} would thus have been more likely to form insoluble complexes. This may explain why FCCP-mediated $[Ca^{2+}]_i$ increases over and above the peak achieved with glutamate were unusual after the 5 min stimulation (Fig. 5*B*). The fact that large pools of FCCP-releasable Ca^{2+} were not routinely demonstrable is consistent with the data indicating that emptying of the matrix Ca^{2+} required tens of minutes following a prolonged glutamate stimulation protocol.

FCCP and CGP-37157 as experimental tools

We have used FCCP as a tool to rapidly and reversibly block mitochondrial Ca^{2+} uptake. Although more selective, Ruthenium Red is not readily membrane permeant and thus not suitable for the current set of experiments. FCCP, however, is not a specific inhibitor of mitochondrial Ca^{2+} uptake and has a number of other effects as a protonophore. It dissipates the pH gradient, depolarizes the mitochondria, and accelerates the rate of electron transport. At concentrations similar to those used in this study, FCCP will also acidify central neurones (Wang, Richardson & Thayer, 1995) by promoting membrane potential-dependent H^+ influx. Finally, FCCP treatment will, under certain conditions, result in the loss of cellular ATP since the oligomycin-sensitive, mitochondrial F_1–F_0-ATPase can reverse direction and consume cellular ATP. Previous results suggest that there is no measurable loss of ATP under the conditions employed here (White & Reynolds, 1995; Wang & Thayer, 1996).

Budd & Nicholls (1996) report that FCCP treatment of central neurones causes a drop in the ATP/ADP ratio. It is this ratio which dictates the thermodynamic favourability of most ATP-dependent reactions, including those governing Ca^{2+} homeostasis. They used rotenone to inhibit electron transport but preserved ATP/ADP with oligomycin; under these circumstances, the membrane potential would be dissipated and Ca^{2+} uptake blocked. Using this method to inhibit mitochondria, they demonstrate that 50 mM K^+ stimulation does not produce the larger $[Ca^{2+}]_i$ transients that would have been predicted based upon our earlier report. Their data describe important problems with FCCP, and the authors conclude that mitochondrial Ca^{2+} uptake plays a minimal role in neuronal Ca^{2+} buffering. This conclusion stands in contrast to a significant body of work in different preparations (Thayer & Miller, 1990; Stuenkel, 1994; Kiedrowski & Costa, 1995; White & Reynolds, 1995; Wang & Thayer, 1996). In particular, Wang, Randall & Thayer (1994) have used Ruthenium Red to complement FCCP as a tool. They demonstrated that important metabolic consequences of glutamate stimulation were different when mitochondrial Ca^{2+} uptake was blocked with this more selective compound.

Several important differences in experimental approach make it difficult to compare the results of Budd & Nicholls (1996) with those described here. Firstly, we did not apply FCCP until after the glutamate stimulus and yet we were able to observe effects almost immediately (Fig. 4*B*). Since we could not detect any change in [ATP] during the first 2 min at room temperature (White & Reynolds, 1995), it seems unlikely that loss of ATP was responsible for such rapid changes in $[Ca^{2+}]_i$. Secondly, the two studies were performed at different temperatures. Consistent with most cell culture studies of Ca^{2+} homeostasis (Bleakman *et al.* 1993; Wang *et al.* 1994; Kiedrowski & Costa, 1995), we conducted our experiments at room temperature while those of Budd & Nicholls were at 37 °C. It is conceivable that the various proteins involved in $[Ca^{2+}]_i$ homeostasis are differentially temperature sensitive, thus changing the relative importance of the separate components at physiological temperature. The rate of change in the ATP/ADP ratio could also be affected by this temperature difference. Thirdly, the studies used different culture preparations. We have chosen embryonic rat forebrain neurones while Budd & Nicholls (1996) used cerebellar granule cells. No comprehensive comparison of the two culture systems has been made and it is probably not reasonable to conclude that the dominant Ca^{2+} buffering processes are similar. Additionally, a number of studies have illustrated the fact that glutamate stimulation induces Ca^{2+} loads with characteristics distinct from those triggered by depolarization alone (Hartley *et al.* 1993; Tymianski *et al.* 1993; Eimerl & Schramm, 1994; White & Reynolds, 1995). It is conceivable that the results of Budd & Nicholls (1996) would have been different if they had used glutamate as a stimulus. Finally, the authors demonstrate a drop in ATP/ADP ratio using a protonophore concentration substantially greater than the one we used here (100 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), Budd & Nicholls, 1996). Further experimentation will be necessary to assess the importance of temperature or cell type specific differences in accounting for our disparate results.

Previous findings using cardiac myocytes have suggested that CGP-37157 is a specific inhibitor of mitochondrial Na^+–Ca^{2+} exchange (Cox *et al.* 1993; Cox & Matlib, 1993), but the present experiments are the first to use CGP-37157 in intact cells to address the role of mitochondria in Ca^{2+} homeostasis. This agent provided us with an approach which complemented the use of FCCP to address the issue of mitochondrial Ca^{2+} uptake. The results we obtained are consistent with the production of a specific and reversible inhibition of mitochondrial Na^+–Ca^{2+} exchange with CGP-37157. Two other investigations have attempted to isolate mitochondrial Ca^{2+} efflux by changing the $[Na^+]_i$ (Kiedrowski & Costa, 1995; Wang & Thayer, 1996) and they also conclude that Na^+-dependent Ca^{2+} efflux from mitochondria modifies the recovery of $[Ca^{2+}]_i$ following glutamate stimulation. Our data present important corroboration for theirs since CGP-37157 inhibits the mitochondrial exchange without changing the myriad other processes dependent on intra- and extracellular Na^+. Although the current work does not directly evaluate the specificity of CGP-37157, the data are consistent with the proposed intracellular target of the drug and other work to date suggests that the drug is specific (in cardiac myocytes) at the concentration used (Cox *et al.* 1993).

Mitochondrial Ca^{2+} buffering in physiology and pathophysiology

The experiments in Fig. 4*B* and *D* suggest that even during a 15 s stimulus a very large amount of Ca^{2+} could be accumulated in mitochondria. Substantial variability was observed in the rate of recovery from these brief but intense

stimuli (Fig. 4*E*). Interestingly, it seems that much of the variability which we observed came from differences in the rate of mitochondrial Ca^{2+} efflux, since eliminating mitochondrial efflux with CGP-37157 caused the neurones to adopt a uniform response in nine different experiments. The application of CGP-37157 following a 5 min, 3 μM glutamate stimulus also resulted in a reduction in the between-cell variability, further substantiating the hypothesis that some of this variability stems from the activity of the mitochondrial Na^+–Ca^{2+} exchange.

It is interesting to speculate about the impact of CGP-37157 on neuronal mitochondrial function. McCormack & Denton (1993) have proposed that mitochondrial Ca^{2+} uptake serves as an essential link coupling neuronal activity to mitochondrial energy production. Work from their laboratory and others has demonstrated that elevations in matrix Ca^{2+} ranging from 500 nM to 3 μM serve to activate several metabolic processes. Three key enzymes in the tricarboxylic acid cycle are ultimately regulated by matrix Ca^{2+}; pyruvate dehydrogenase (the rate-limiting step for acetyl CoA synthesis); isocitrate dehydrogenase; and 2-oxoglutarate dehydrogenase. These enzymes will collectively increase the redox potential driving the respiratory chain. In a parallel process, mitochondrial Ca^{2+} uptake results in an expansion of the matrix volume and a subsequent increase in the rate of electron transport. Thus, a higher redox potential (generated by the tricarboxylic acid cycle) drives reducing equivalents through an activated respiratory chain. Finally, in a third independent process, matrix Ca^{2+} activates the oligomycin-sensitive F_1–F_0-ATPase which ultimately converts the proton motive force into cytoplasmic ATP equivalents. Preventing the efflux of Ca^{2+} from the matrix might, therefore, enhance the rate of ATP production. Indeed, it is this mechanism that Cox and colleagues (1993) have suggested to account for the positive inotropic effects induced by CGP-37157. In support of this, we have observed neuronal mitochondrial hyperpolarization following the application of CGP-37157 (White & Reynolds, 1996).

Detrimental processes can also be triggered by large elevations of matrix Ca^{2+}. We have demonstrated that intense glutamate stimulation induces mitochondrial depolarization and further results in the production of reactive oxygen species with mitochondria as the candidate source (Reynolds & Hastings, 1995; White & Reynolds, 1996). Our studies suggest that the permeability transition pore may be activated by glutamate as a result of elevated matrix Ca^{2+}, a process that would be potentiated if the pore were oxidized (Bernardi *et al.* 1994). As permeability transition will result in the loss of the gradient responsible for ATP synthesis, this process may be quite toxic to all cell types. Interestingly, when we studied the effects of glutamate on mitochondrial membrane potential, we found that CGP-37157 promoted mitochondrial hyperpolarization in some cells and depolarization in others. Thus, it is hard to predict whether this drug will enhance or abrogate excitotoxicity in neurones.

The role of $[Na^+]_i$ in Ca^{2+} homeostasis

As a first approximation, it seems intuitive that removal of extracellular Na^+ would undermine the ability of a neurone to recover from a glutamate-induced Ca^{2+} load. Glutamate stimulation will raise both $[Ca^{2+}]_i$ and $[Na^+]_i$ and will also acidify the neuronal cytoplasm (Hartley & Dubinsky, 1993; Koch & Barish, 1994; Irwin, Lin, Long & Paul, 1994; Kiedrowski & Costa, 1995; Stout *et al.* 1996). Since there are Na^+-dependent exchangers on the plasma membrane for both H^+ and Ca^{2+}, it seems obvious that removal of extracellular Na^+ will necessarily compromise the recovery of the cell following an intense glutamate challenge. Koch & Barish (1994) describe marked changes in $[Ca^{2+}]_i$ recovery dynamics following removal of extracellular Na^+ and were also able to differentiate the effects on Na^+–Ca^{2+} exchange from those on Na^+–H^+ exchange. Obviously, decreasing extracellular Na^+ will also facilitate loss of $[Na^+]_i$ and Wang and Thayer (1996) have shown that reducing $[Na^+]_i$ dramatically slows the rate of mitochondrial Ca^{2+} efflux.

However, the interplay between $[Na^+]_i$ and $[Ca^{2+}]_i$ is complex. In their experiments, slowing the rate of mitochondrial Ca^{2+} efflux delayed the overall recovery of $[Ca^{2+}]_i$ because intracellular Na^+ depletion only reduced mitochondrial Ca^{2+} efflux (Wang & Thayer, 1996). Thus Ca^{2+} trickled out of the mitochondria over a prolonged period. Since CGP-37157 allowed us to block mitochondrial Ca^{2+} efflux altogether, we observed a rapid drop to baseline and then a later rise and fall after the drug was washed out (Figs 4*D* and 5*D*). Both experimental protocols highlight a previously underappreciated role for $[Na^+]_i$ in Ca^{2+} homeostasis. Mitochondrial Na^+–Ca^{2+} exchange occurs at the expense of ATP generation (Gunter *et al.* 1994; Wang *et al.* 1994) and uptake of a Ca^{2+} ion which is only going to be extruded in a Na^+-dependent fashion (so called 'Ca^{2+}-cycling') is a waste of cellular energy. Normally, this loss of ATP is an acceptable cost for coupling matrix $[Ca^{2+}]_i$ with that in the cytoplasm, presumably for the above-mentioned purpose of relating ATP production with cellular activity. However, the circumstances of excitotoxic glutamate stimulation are precisely the time when such a system will be undesirable: Ca^{2+} continues to flood the neuronal cytoplasm, Ca^{2+} cycles in and out of the mitochondria at the expense of ATP production and the plasma membrane extrusion mechanisms are overwhelmed.

In conclusion, this report represents an important step in our understanding of the mechanisms regulating $[Ca^{2+}]_i$ following glutamate receptor activation. As the stimuli used in this study approach those normally expected to be toxic to neurones, these studies also highlight the critical components likely to be involved in the determination of whether a given glutamate stimulus will prove to be lethal or not. Our experiments may thus represent a starting point for understanding the basis of the mechanism of action of growth factors and other peptides that modulate the induction or expression of excitotoxicity (Cheng & Mattson, 1991; Prehn, Bindokas, Marcuccilli, Krajewski, Reed &

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Author's email address

I. J. Reynolds: ijr@prophet.pharm.pitt.edu

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