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Cytosolic Ca^{2+} Changes during *In Vitro* Ischemia in Rat Hippocampal Slices: Major Roles for Glutamate and Na^+-Dependent Ca^{2+} Release from Mitochondria

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This work determined Ca^{2+} transport processes that contribute to the rise in cytosolic Ca^{2+} during *in vitro* ischemia (deprivation of oxygen and glucose) in the hippocampus. The CA1 striatum radiatum of rat hippocampal slices was monitored by confocal microscopy of calcium green-1. There was a 50–60% increase in fluorescence during 10 min of ischemia after a 3 min lag period. During the first 5 min of ischemia the major contribution was from Ca^{2+} entering via NMDA receptors; most of the fluorescence increase was blocked by MK-801. Approximately one-half of the sustained increase in fluorescence during 10 min of ischemia was caused by activation of Ca^{2+} release from mitochondria via the mitochondrial $2Na^+$–Ca^{2+} exchanger. Inhibition of Na^+ influx across the plasmalemma using lidocaine, low extracellular Na^+, or the AMPA/kainate receptor blocker CNQX reduced the fluorescence increase by 50%. The $2Na^+$–Ca^{2+} exchange blocker CGP37157 also blocked the increase, and this effect was not additive with the effects of blocking Na^+ influx. When added together, CNQX and lidocaine inhibited the fluorescence increase more than CGP37157 did. Thus, during ischemia, Ca^{2+} entry via NMDA receptors accounts for the earliest rise in cytosolic Ca^{2+}. Approximately 50% of the sustained rise is attributable to Na^+ entry and subsequent Ca^{2+} release from the mitochondria via the $2Na^+$–Ca^{2+} exchanger. Sodium entry is also hypothesized to compromise clearance of cytosolic Ca^{2+} by routes other than mitochondrial uptake, probably by enhancing ATP depletion, accounting for the large inhibition of the Ca^{2+} increase by the combination of CNQX and lidocaine.

Key words: NMDA; AMPA/kainate; mitochondria; CGP37157; sodium channels; CNQX

Cell calcium is thought to play a key role in mediating ischemic neuronal damage (for review, see Sweeney et al., 1995; Kristian and Siesjo, 1996), and thus it is critical to understand how it is regulated. Cytosolic Ca^{2+} is elevated during global ischemia (Erecinska and Silver, 1992; Nakamura et al., 1999) and during *in vitro* ischemia (deprivation of glucose and oxygen) in brain slices (Mitani et al., 1993), as well as in neuronal cell cultures that are exposed to mitochondrial and glycolytic inhibitors (Dubinsky and Rothman, 1991). Ischemia induces a large increase in glutamate release in brain tissue *in vivo* (Benveniste et al., 1984) and *in vitro* (Lobner and Lipton, 1990), and there is evidence of an NMDA component to the increase in cytosolic Ca^{2+} during global ischemia (Erecinska and Silver, 1992), during anoxia in cortical slices (Bickler and Hansen, 1994), and possibly in organotypic hippocampal cultures (Velazquez et al., 1997). Other than this, nothing is known about the pathways that mediate the ischemic increases in cytosolic Ca^{2+}.

The present work, using the rat hippocampal slice, was designed to understand more fully the mechanisms by which cytosolic Ca^{2+} is regulated during ischemia. Free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) changes in s. radiatum of the CA1 region were monitored using confocal fluorescent microscopy of the Ca^{2+} indicator calcium green-1. The roles of both Ca^{2+} influx and its release from internal stores (particularly, mitochondria) were assessed. Sodium entry-mediated activation of the mitochondrial $2Na^+$–Ca^{2+} exchanger seems to play a major role in regulating cytosolic Ca^{2+} during ischemia. This has not, heretofore, been recognized.

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MATERIALS AND METHODS

Slice preparation. Transverse hippocampal slices were prepared as described previously (Kass and Lipton, 1982). Adult male Sprague Dawley rats (225–250 gm) were decapitated. The rat brain was rapidly removed and put into ice-cold modified standard buffer (see below), The hippocampi were isolated, and transverse slices (300 μm thick) were sectioned with a vibratome (Telios Pharmaceuticals Inc., San Diego, CA). Slices were then incubated in modified standard buffer for 45 min at 33°C and transferred to standard buffer for 75 min before any experiment. All experiments were performed at 37°C.

Buffers. Standard buffer was 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.4 mM KH_2PO_4, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2$, and 10 mM glucose. Buffers were equilibrated with 95% O_2/5% CO_2, pH 7.4. Modified standard buffer was the same as standard buffer except for 10 mM $MgSO_4$ and 0.5 mM $CaCl_2$. "Ischemic" buffer was the same as standard buffer but without glucose and equilibrated with 95% N_2/5% CO_2. A 20 min equilibration with ischemic buffer reduced the oxygen saturation to <1%. In Ca^{2+}-free (0-Ca^{2+}) buffer (standard or ischemic), $CaCl_2$ was omitted from the above buffers, and 200 μM EGTA was added. For experiments performed in this buffer, slices were incubated for 20 min before ischemia. In low Na^+ buffer, NaCl was replaced with *N*-methyl-D-glucamine; $NaHCO_3$ was still present.

Free $[Ca^{2+}]_i$ measurement with fluorescent confocal microscopy. The change in $[Ca^{2+}]_i$ was measured with the long wavelength calcium indicator calcium green-1 AM (Molecular Probes, Eugene, OR). Fresh solutions of 1 mM calcium green-1 AM were made in dehydrated DMSO before each experiment, and hippocampal slices were immersed in the standard buffer containing a final concentration of 10 μM calcium green-1

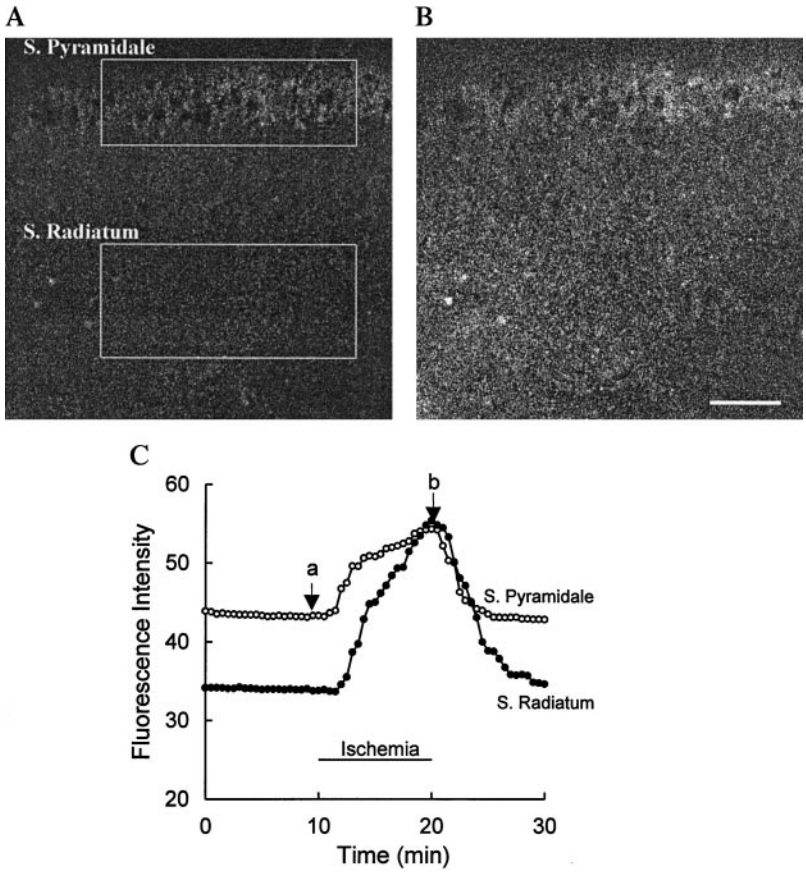

Figure 1. Ca^{2+} fluorescence before and during ischemia. Rat hippocampal slices (300 μm) were loaded with 10 μM calcium green-1 AM for 45 min at 33°C and washed in normal buffer for at least 30 min before the experiments. *In vitro* ischemia was achieved by switching the normal buffer to glucose-free buffer equilibrated with 95% N_2/5% CO_2. *A*, *B*, Representative images taken in the CA1 area before and at the end of 10 min of ischemia, respectively. Scale bar, 100 μm. *C*, One typical recording of Ca^{2+} fluorescence during and after ischemia (*horizontal line*) from s. pyramidale and s. radiatum highlighted by *rectangles* in *A*. There was no correction for photo bleaching (curve-fitting) in this experiment. *Points a* and *b* represent the time points for *A* and *B*, respectively. The increase in fluorescence intensity was larger in s. radiatum than in s. pyramidale.

AM for 45 min at 33°C. The loading temperature was set at 33°C instead of 37°C to reduce potential compartmentalization of the dye in the cell (Roe et al., 1990). Pluronic (0.1%) was added to the incubation medium because it increases the solubility of the highly hydrophobic dye and thus increases the loading of the cells with the dye. The slices were then placed back in fresh standard buffer for at least 30 min to wash away extracellular dye. This treatment did not affect the slice viability as measured by extracellular recordings of synaptic transmission between CA3 and CA1 regions. Population spike height and latency did not differ between control and dye-loaded slices ($n = 3$; data not shown).

The slice was put on a coverslip in a closed perfusion chamber and held still by a nylon net glued to a U-shaped platinum wire. Gas-equilibrated buffer was maintained at 37°C in reservoirs and was used to perfuse the slice in a recirculating system. The perfusion rate was set at ~20 ml/min by adjusting the diameter of a fine pipette tip attached to the perfusion chamber. The buffer volume in the chamber was 0.5 ml so that turnover was very rapid. Buffer exchange in the chamber was achieved by a switch attached to the two buffer reservoirs, and it took 30 sec to completely replace one buffer with another as measured with an oxygen probe.

The perfusion chamber was placed on the stage of a Nikon diavert microscope. The optical recording system included an inverted Nikon Diaphot 200 microscope equipped with an argon laser source and a confocal system [both a Noran and a Bio-Rad (Hercules, CA) 1000 system were used in this study]. The slice was viewed under a 20× objective with a numerical aperture of 4.0 and a working distance of ~300 μm from the optical section. Laser intensity was set at 3–10% of the maximum level to help reduce dye bleaching and to protect the slices against possible photo damage. However, this low level provided a substantial baseline signal, shown in Figure 1*C*. The laser intensity and scale were adjusted so that basal fluorescence intensity in arbitrary units was 35–45 on a scale whose maximum was 255. This avoided any saturation during ischemia. Calcium green-1 was excited at 488 nm, and the emission was measured at 510 nm. Images were saved on the hard drive and analyzed with the Time Course program from Bio-Rad.

The CA1 area of the hippocampus was localized using the normal light microscope mode. Fluorescent signals from s. radiatum of the CA1 region were collected between 80 and 100 μm from the bottom surface of the slice. The slice was 300 μm thick, so this was ~200 μm from the perfused surface of the slice.

The field of view in s. radiatum was carefully chosen to avoid any cell bodies, astrocytes, interneurons, or blood vessels. Electron micrographs of typical fields showed that the region had the following composition by area: dendrites, 53%; presynaptic elements, 33%; and unidentified, 14%.

All experiments used paired experimental and control slices from the same hippocampus. Each paradigm consisted of four to eight such pairs, and each pair of slices in a particular paradigm came from a different rat.

Data analysis. Calcium green-1 is not a ratiometric indicator; thus changes in $[Ca^{2+}]_i$ levels could only be expressed as the percentage changes in fluorescence over the baseline $[(\Delta F/F) \times 100]$ and not as changes in absolute Ca^{2+} concentrations. The slow bleaching of the dye was corrected using a curve-fitting program. Readings in control conditions, taken for 10 min before the onset of ischemia, were fitted to a curve (with Microsoft Excel) that was extrapolated into the ischemic period and considered to represent the baseline during that period and the reoxygenation period. Those extrapolated values were subtracted from the actual reading to give the net fluorescence change during ischemia or reoxygenation periods. There was, in fact, very little photo bleaching at these low laser intensities, as shown in Figure 1*C*, in which no correction was made. All values in the bar graphs are means ± SEM. Student's *t* test (for two groups) and ANOVA followed by Dunnett's test (for three or more groups) were used to determine the statistical significance of any differences.

Materials. Calcium green-1 AM was from Molecular Probes; MK-801 and CNQX were purchased from Research Biochemicals (Natick, MA); CGP37157 was a gift from CIBA (Suffren, NY); GYKI52466 was from Research Biochemicals; and all other chemicals were from Sigma (St. Louis, MO). Stock solutions were made in the following ways: CNQX (20 mM), GYKI52466 (3 mM), MK-801 (10 mM), nimodipine (100 mM), nifedipine (100 mM), and benzamil (100 mM) were in ethanol; lidocaine (50 mM) was in deionized distilled water; and CGP37157 (10 mM) was in DMSO. All agents were added to the buffer 30 min before ischemia unless stated otherwise. None of them affected the basal fluorescence under normoxia in preliminary control studies.

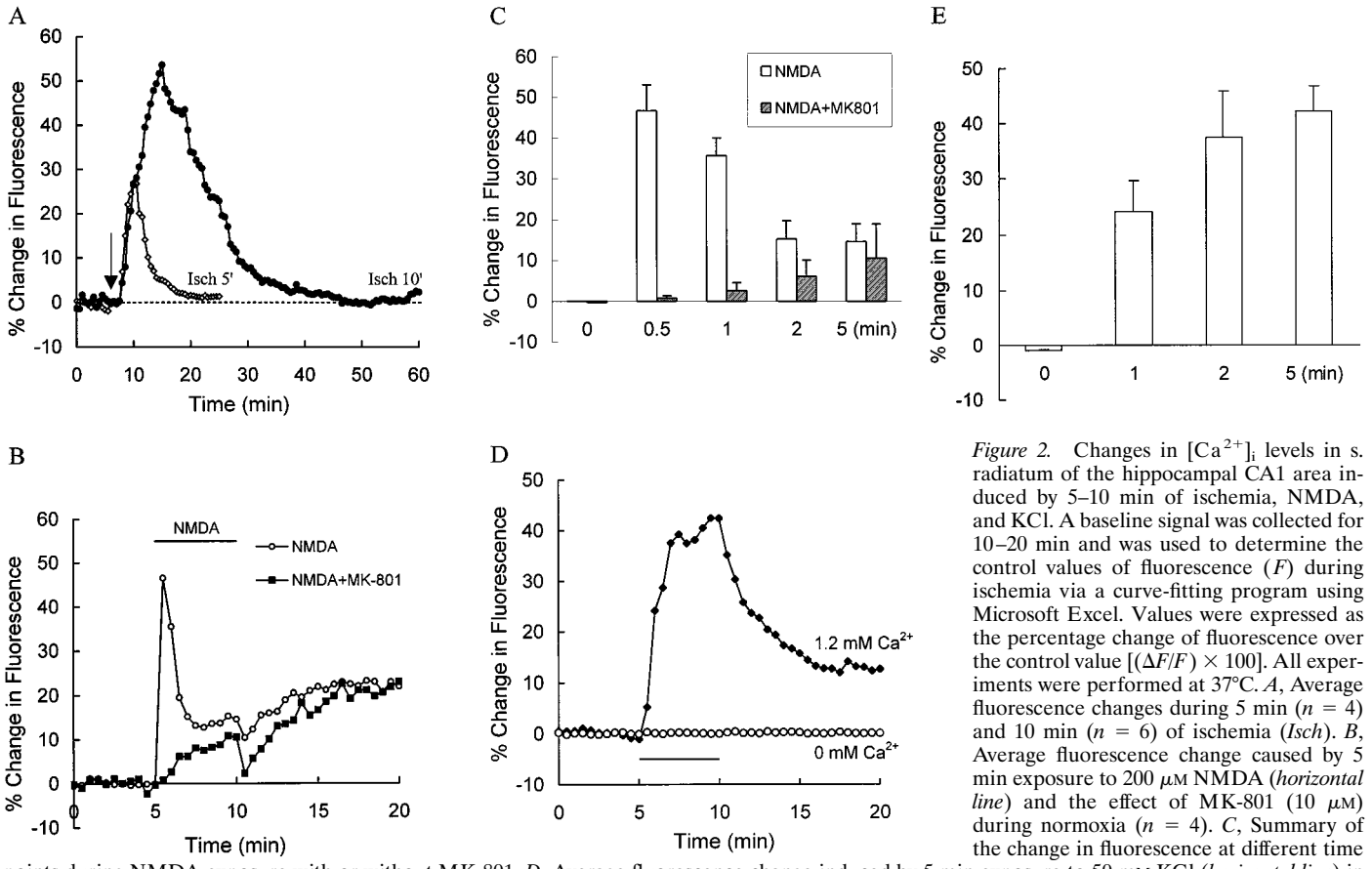

Figure 2. Changes in $[Ca^{2+}]_i$ levels in s. radiatum of the hippocampal CA1 area induced by 5–10 min of ischemia, NMDA, and KCl. A baseline signal was collected for 10–20 min and was used to determine the control values of fluorescence (F) during ischemia via a curve-fitting program using Microsoft Excel. Values were expressed as the percentage change of fluorescence over the control value $[(\Delta F/F) \times 100]$. All experiments were performed at 37°C. *A*, Average fluorescence changes during 5 min ($n = 4$) and 10 min ($n = 6$) of ischemia (*Isch*). *B*, Average fluorescence change caused by 5 min exposure to 200 μM NMDA (*horizontal line*) and the effect of MK-801 (10 μM) during normoxia ($n = 4$). *C*, Summary of the change in fluorescence at different time points during NMDA exposure with or without MK-801. *D*, Average fluorescence change induced by 5 min exposure to 50 mM KCl (*horizontal line*) in 1.2 mM Ca^{2+} ($n = 4$) or Ca^{2+}-free ($n = 3$) buffer. *E*, Summary of KCl effects in normal Ca^{2+} buffer at different time points of exposure. Note that the lag time for the rise of $[Ca^{2+}]_i$ was much shorter (30 sec) in the presence of NMDA or KCl than during ischemia (~3 min).

RESULTS

Changes in $[Ca^{2+}]_i$ after *in vitro* ischemia compared with changes caused by NMDA or high extracellular K^+

Rat hippocampal slices were exposed to either 5 or 10 min of ischemia followed by different reoxygenation periods (up to 120 min). Figure 1, *A* and *B*, shows representative images of fluorescence in the CA1 area before and during ischemia, respectively. The absolute change in fluorescence intensity during ischemia is less prominent in s. pyramidale than in s. radiatum (Fig. 1*C*). The present study focused on the Ca^{2+} transient in CA1 s. radiatum because there are several Ca^{2+}-regulated cytoskeletal changes in dendrites, including microtubule dissolution and microtubule-associated protein 2 (MAP2) breakdown (Y. Zhang and P. Lipton, unpublished observations). Figure 2*A* shows the fluorescence response in s. radiatum to 5 and 10 min of ischemia. There were significant increases in $[Ca^{2+}]_i$ during 5–10 min of ischemia, which began after an average lag time of ~3 min and were dependent on the duration of ischemia. The fluorescence change was 30% at the end of 5 min of ischemia and ~55% at the end of 10 min of ischemia.

The half-time required for the $[Ca^{2+}]_i$ return to baseline during the reoxygenation period was 2.7 ± 0.4 and 8.8 ± 0.83 min for 5 and 10 min of ischemia, respectively. It took 30–50 min of reoxygenation for intracellular Ca^{2+} to return fully to the baseline after 10 min of ischemia.

The $[Ca^{2+}]_i$ change induced by ischemia was compared with that induced by NMDA and by depolarizing the cell with KCl. As shown in Figure 2*B*, 200 μM NMDA caused a rapid transient increase in fluorescence of approximately the same magnitude as that caused by ischemia. There was also a delayed, smaller fluorescence increase. Both these changes were blocked by MK-801. A slower change occurred in the presence of MK-801; this change was not significant as shown in Figure 2*C*. It resulted from a change in one of four experiments. Increasing extracellular K^+ to 50 mM also increased $[Ca^{2+}]_i$ transiently, by approximately the same amount as ischemia (Fig. 2*D*,*E*). This effect was dependent on extracellular Ca^{2+} because there was no change in Ca^{2+} fluorescence in the absence of extracellular Ca^{2+} (Fig. 2*D*). This observation provides strong supporting evidence that changes in fluorescence reflect changes in $[Ca^{2+}]_i$ and are not caused by changes in cell volume, because cells swell significantly in KCl buffer, whether or not Ca^{2+} is present (Andrew and MacVicar, 1994; Takahashi et al., 1995). Although the magnitudes of the changes were similar to those during ischemia, there was only an ~30 sec lag in the elevation of $[Ca^{2+}]_i$ induced by either NMDA or KCl. Thus, the 3 min lag in the $[Ca^{2+}]_i$ rise during ischemia was not an artifact caused by the delay in exchanging buffer.

Lag time for the $[Ca^{2+}]_i$ rise is reduced when Cl^- is substituted by an impermeant anion

The lag time, which has been noted by others (Mitani et al., 1993), was unexpected because $^{45}Ca^{2+}$ entry occurs during the first 2.5 min of ischemia (Lobner and Lipton, 1993). Its origin was investigated. Cerebral tissue, including the brain slice, undergoes rapid cellular swelling during anoxia or ischemia (Lipton, 1973). It was

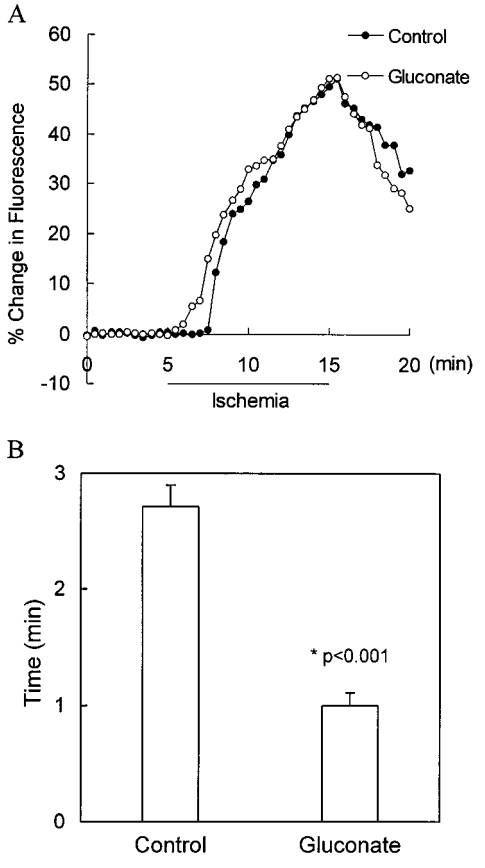

Figure 3. Effect of replacing Cl^- with gluconate on $[Ca^{2+}]_i$ during ischemia. *A*, There was normally a 2.5–3.5 min delay in the onset of fluorescence change (control curve). Replacing extracellular Cl^- did not affect the basal fluorescence but resulted in a much earlier fluorescence increase. The fluorescence rose as early as 0.5 min after the onset of ischemia. *B*, The average lag time for both control and gluconate-treated slices is shown. The latter was significantly different from the control condition ($*p < 0.001$; $n = 6$ for each trace).

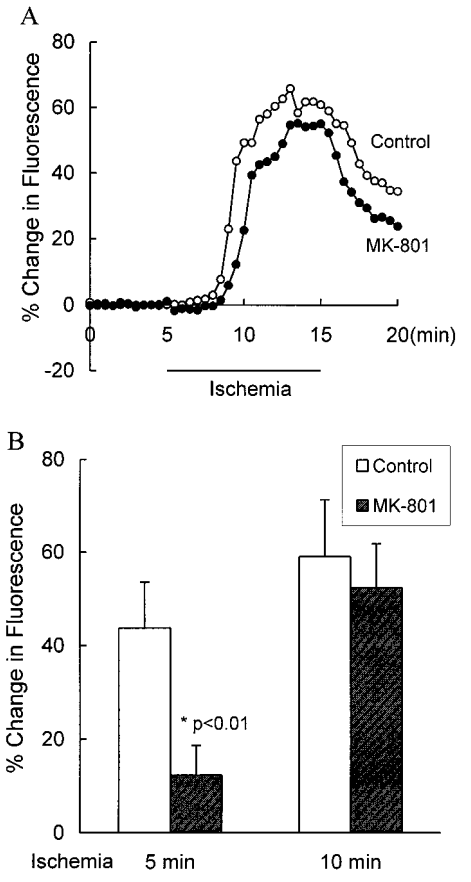

Figure 4. Effect of MK-801 on $[Ca^{2+}]_i$ during 10 min of ischemia. MK-801 (10 μM) was added to the buffer 30 min before and during ischemia. *A*, Average traces of fluorescence for the control and MK-801 groups during 10 min of ischemia (*horizontal line*). *B*, The change in fluorescence levels after 5 and 10 min of ischemia with or without MK-801. MK-801 reduced the $\Delta F/F$ from 43.7 ± 9.7 to 12.3 ± 6.3% at 5 min of ischemia but showed no significant effect at the end of 10 min of ischemia ($n = 6$).

reasoned that the lag in the increase in cytosolic free Ca^{2+} might result from a dilution of the entering Ca^{2+} by the increased cellular water volume. The cell swelling was prevented by incubating the slices in buffer in which Cl^- was replaced by the gluconate anion, which is impermeant and prevents iso-osmotic cell swelling (Lipton, 1973; MacVicar and Hochman, 1991). As shown in Figure 3, the lag time was reduced to ~1 min in this case, suggesting that the normal lag does result from cell swelling.

Role of Ca^{2+} influx across the plasmalemma in the rise in $[Ca^{2+}]_i$: importance of NMDA receptors

The role of NMDA-type glutamate receptors in the $[Ca^{2+}]_i$ increase during ischemia was tested using 10 μM MK-801, a noncompetitive blocker of the NMDA receptor. Figure 4*A* shows that MK-801 significantly delayed the elevation of $[Ca^{2+}]_i$. As summarized in Figure 4*B*, the drug reduced the change in $[Ca^{2+}]_i$ at 5 min but had no effect at 10 min, indicating that the influx of Ca^{2+} through NMDA receptors contributes to the early increase in intracellular calcium but not to the later accumulation. MK-801 similarly delayed the $[Ca^{2+}]_i$ increase during ischemia in the gluconate buffer (data not shown).

The roles of several other potential calcium influx pathways in the $[Ca^{2+}]_i$ elevation were examined using specific blockers (Fig. 5). L-type Ca^{2+} calcium channel blockers nimodipine and nifedipine (data not shown), the N-type Ca^{2+} channel blocker ω-conotoxin GVIA, and the plasmalemma $3Na^+$–Ca^{2+} exchange blocker benzamil all failed to alter the Ca^{2+} transient significantly. As shown in Figure 5, nimodipine greatly attenuated the elevation of cytosolic Ca^{2+} induced by 50 mM KCl under normoxic conditions, demonstrating the efficacy of the drug in our system. Thus, although some of these pathways contribute to the net Ca^{2+} entry (Lobner and Lipton, 1993), they do not contribute to the rise in cytosolic Ca^{2+}. The reason for this is unknown. One possibility is that Ca^{2+} entering across the dendritic plasma membrane is rapidly taken up by the mitochondria, which are close to the plasmalemma in small dendrites. This occurs in endothelial cells in which mitochondria are proximal to the plasmalemma (Lawrie et al., 1996).

Role of mitochondria in the rise of $[Ca^{2+}]_i$

The transient effect of NMDA blockade on $[Ca^{2+}]_i$ and the absence of any effect of Ca^{2+} channel blockers or the $3Na^+$–Ca^{2+} exchange blocker indicate that a source other than extracellular Ca^{2+} was responsible for the majority of the rise in $[Ca^{2+}]_i$ during ischemia. The mitochondria constitute a source of Ca^{2+} that could, in principle, be tapped if the $2Na^+$–Ca^{2+} exchanger in its inner membrane, which mediates Ca^{2+} efflux

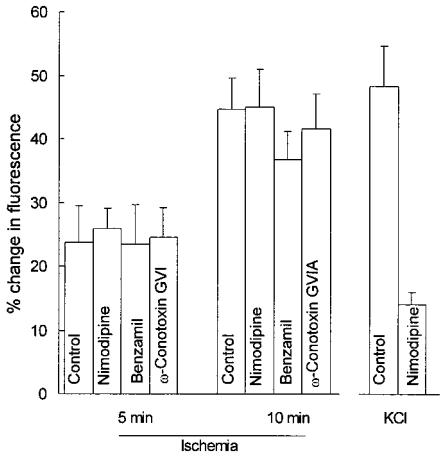

Figure 5. *Left*, *Middle*, Effects of nimodipine, ω-conotoxin GVIA, and benzamil on $[Ca^{2+}]_i$ during 5 and 10 min of ischemia are shown. None of the drugs inhibited the fluorescence increase during ischemia [$n = 5$, 4, and 6 for nimodipine (20 μM), ω-conotoxin GVIA (2 μM), and benzamil (100 μM) experiments, respectively]. *Right*, Rat hippocampal slices were exposed to 50 mM KCl for 5 min under nonischemic conditions. Nimodipine significantly suppressed the elevation of cytosolic Ca^{2+} during KCl exposure, demonstrating the efficacy of this drug in the present system.

from the mitochondria (Gunter and Pfeiffer, 1990), was activated by increased cytosolic Na^+. In fact there is a significant increase in intracellular Na^+ within 5 min of anoxia in rat hippocampal slices (Fried et al., 1995) (see below), which should activate this exchanger. This possibility was tested in a series of studies that are shown in Figures 6 and 7.

Blockade of the mitochondrial $2Na^+$–Ca^{2+} exchanger reduced the $[Ca^{2+}]_i$ elevation during ischemia

CGP37157 is a specific blocker of the mitochondrial $2Na^+$–Ca^{2+} exchanger in heart cells (Cox et al., 1993) and blocks the exchanger in neurons and phenochromocytoma-12 cells also (Baron and Thayer, 1997; White and Reynolds, 1997). At 10 μM, this drug reduced the rise in $[Ca^{2+}]_i$ from 48.9 ± 7.2 to 26.2 ± 5.7% ($p < 0.05$) at the end of 10 min of ischemia (Fig. 6*A*), suggesting the importance of Na^+-induced Ca^{2+} release from the mitochondria.

Reduction in Na^+ entry attenuated the $[Ca^{2+}]_i$ increase during ischemia

Because the mitochondrial $2Na^+$–Ca^{2+} exchanger is activated by elevated cytosolic Na^+, it seemed probable that blocking Na^+ entry during ischemia would attenuate the release of mitochondria Ca^{2+} and hence the increase in cytosolic Ca^{2+}. This hypothesis was tested in two sets of experiments.

In the first set of experiments two different AMPA receptor blockers, CNQX and GYKI52466 (Fig. 6*B*), or the sodium channel blocker lidocaine (Fig. 6*C*), which blocks depolarization-sensitive Na^+ channels and also blocks glutamate release during ischemia (Taylor et al., 1995), were added to reduce Na^+ entry. In both cases the steady-state rise in $[Ca^{2+}]_i$ was attenuated by ~50%. Lidocaine also severely attenuated the early increase in $[Ca^{2+}]_i$, probably because it blocks glutamate release and hence the NMDA receptor-mediated early Ca^{2+} entry. Including both CNQX and lidocaine in the buffer inhibited the $[Ca^{2+}]_i$ elevation during 10 min of ischemia further than either drug alone (Fig. 6*D*) and further than CGP37157. This suggests effects beyond simply blocking mitochondrial Ca^{2+} release and is considered in the Discussion. The effect of combining MK-801 and CNQX was also additive. The combination significantly delayed the early rise of $[Ca^{2+}]_i$ and also suppressed the late change in $[Ca^{2+}]_i$ during ischemia (Fig. 6*E*).

In the second set of experiments, the role of extracellular Na^+ entry during ischemia was tested nonpharmacologically by reducing Na^+ in the buffer. As shown in Figure 6*F*, reduction of extracellular Na^+ from 150 to 26 mM with the concomitant substitution by *N*-methyl-D-glucamine decreased the $[Ca^{2+}]_i$ elevation during ischemia to the same extent as did CNQX or lidocaine (from 42.2 ± 3.8 to 28.8 ± 3.3% at 10 min).

The effect of CGP37157 on the $[Ca^{2+}]_i$ elevation during ischemia was not additive with the effect of reduced Na^+ entry

When CGP37157 was included with CNQX, inhibition of release was the same as with either drug alone; there was no additivity (Fig. 6*B*). As with CNQX, adding CGP37157 in low Na^+ buffer did not have any further effect (Fig. 6*F*). These results support the hypothesis that Ca^{2+} release from mitochondria is the basis for the action of Na^+ influx on cytosolic Ca^{2+}.

Effects of different treatments on Na^+ influx during ischemia

To test whether CGP37157 might be acting on Na^+ influx rather than on mitochondrial $2Na^+$–Ca^{2+} exchange, we measured the effect of CGP37157 on intracellular Na^+ levels in the whole hippocampal slice during ischemia. As shown in Figure 7*A*, 7.5 min of ischemia increased intracellular Na^+ concentration by ~70%. Adding CGP37157 showed little effect on this increase, indicating that the drug did not block ischemic Na^+ entry.

We also tested whether lidocaine and CNQX inhibited the Na^+ changes during ischemia. The increase in cell Na^+ was strongly inhibited by 50 μM lidocaine, consistent with a previous report of lidocaine's effect under anoxic conditions (Fried et al., 1995). However, despite its large inhibition of the rise in $[Ca^{2+}]_i$, CNQX did not attenuate the increase in intracellular Na^+ (Fig. 7*A*). It was reasoned that because the majority of AMPA receptors are in dendrites, the effect on sodium might be seen in tissue samples exclusively from the neuropil. However, as shown in Figure 7*B*, CNQX did not inhibit the Na^+ increase in neuropil tissue either. This result is considered in the Discussion.

$[Ca^{2+}]_i$ changes during ischemia in 0-Ca^{2+} buffer

There were two reasons for measuring $[Ca^{2+}]_i$ changes during ischemia in 0-Ca^{2+} buffer. The first was to confirm the role of intracellular stores in the $[Ca^{2+}]_i$ increase, and the second was to eliminate the possibility that CNQX and CGP37157 were acting directly on Ca^{2+} entry. The 0-Ca^{2+} conditions in the nominally Ca^{2+}-free buffer were verified by the fact that the high K^+ buffer-induced fluorescence increase was blocked by removal of extracellular Ca^{2+} (Fig. 2*D*).

Figure 8*A* shows that there is a significant increase in $[Ca^{2+}]_i$ during ischemia in the 0-Ca^{2+} buffer, which is similar to that in 1.2 mM Ca^{2+} medium. Such an increase in 0-Ca^{2+} buffer has been noted previously in slices (Mitani et al., 1993).

Effects of different manipulations are summarized in Figure 8*B*. As expected in the absence of extracellular Ca^{2+}, the NMDA antagonist MK-801 had no effect on the rise in $[Ca^{2+}]_i$. However, effects of other agents were very similar to those in normal Ca^{2+} buffer.

CGP37157 inhibited the elevation of $[Ca^{2+}]_i$ during ischemia by ~50% as it did in normal buffer, indicating that mitochondria are a major source of Ca^{2+}. This effect also confirmed that

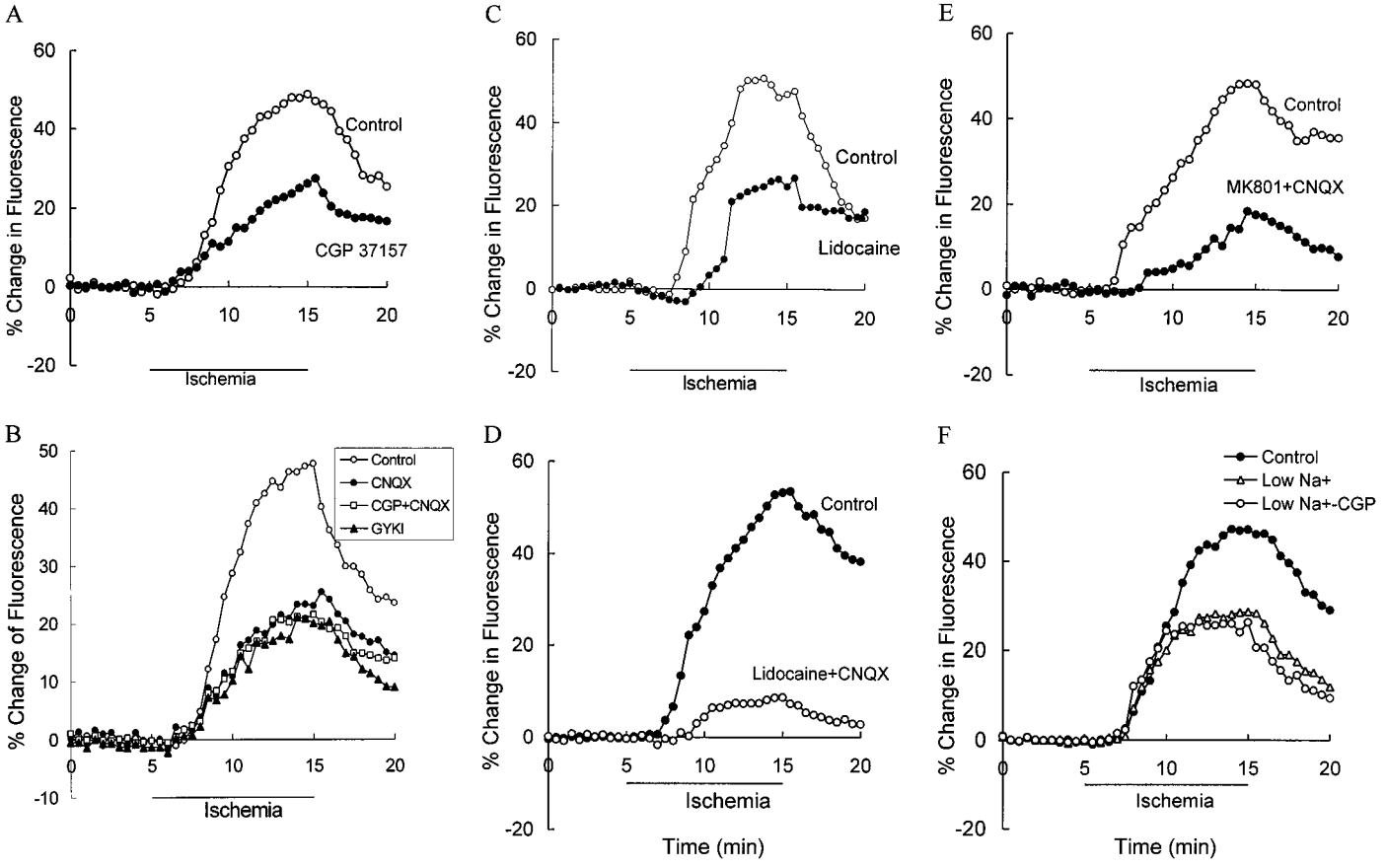

Figure 6. Effects of various drugs and low Na^+ buffer on $[Ca^{2+}]_i$ during ischemia (*horizontal line*). *A*, CGP37157 (10 μM). *B*, CNQX (10 μM) with and without CGP37157 (*CGP*) and GYKI52466 (*GYKI*; 50 μM). *C*, Lidocaine (50 μM). All drugs reduced the fluorescence increase during 10 min of ischemia. *D*, Combination of CNQX and lidocaine showing a stronger inhibition of the fluorescence increase during ischemia than is seen with either drug alone ($n = 6$). *E*, The additive effects of MK-801 (10 μM) and CNQX (10 μM). *F*, Low external Na^+. NaCl in the buffer was replaced with *N*-methyl-D-glucamine. This substitution reduced the fluorescence increase during ischemia, and CGP37157 had no further effect ($n = 6$).

CGP37157 was acting on the mitochondrial exchanger and not on Ca^{2+} influx pathways (Baron and Thayer, 1997).

To see whether the Ca^{2+} increase in 0-Ca^{2+} buffer was dependent on Na^+ entry, we included lidocaine, CNQX, and the combination of the two during the ischemia. As in normal Ca^{2+} buffer, each of the two drugs alone strongly inhibited the increase in $[Ca^{2+}]_i$, and the combination of the two drugs reduced the maximal fluorescence increase even further, to 8% above the baseline. Low Na^+ medium also greatly reduced the elevation in $[Ca^{2+}]_i$, and CGP37157 did not show any further effect on $[Ca^{2+}]_i$ in the low Na^+ solution. The efficacy of CNQX in the 0-Ca^{2+} buffer shows that its effect is not on Ca^{2+} fluxes through AMPA/Kainate channels and thus supports the suggestion that it acts on $[Ca^{2+}]_i$ as a result of blocking Na^+ entry.

Thus fluorescence responses in 0-Ca^{2+} buffer were the same as those in normal Ca^{2+} buffer, suggesting the importance of the Na^+-induced Ca^{2+} release from mitochondria as a source of the cytosolic Ca^{2+} increase. Fifty percent of the steady-state rise in $[Ca^{2+}]$ in both 0-Ca^{2+} and normal buffer was not accounted for as release from mitochondria, although this remaining rise could be largely abolished when CNQX and lidocaine were used together. At least in 0-Ca^{2+} buffer, this rise was not caused by release of Ca^{2+} from mitochondria via the mitochondrial transition pore. Cyclosporin A (10 μM) had no effect on the Ca^{2+} increase in 0-Ca^{2+} buffer (data not shown).

It is notable that there was a very rapid increase in fluorescence even in 0-Ca^{2+} buffer, despite the absence of NMDA receptor-mediated Ca^{2+} entry. The basis for this is very probably the more rapid entry of Na^+ in 0-Ca^{2+} buffer because of increased cell excitability, as manifested by the much more rapid fall in ATP levels in this buffer (Lobner and Lipton, 1993).

DISCUSSION

Calcium green-1 is not a ratiometric dye. Furthermore, its high affinity for Ca^{2+} (~190 nM) means that even large differences in $[Ca^{2+}]_i$ in the range of 300 nM or more will be hard to discern (Hyrc et al., 1997). Thus the importance of the results presented here is that they demonstrate qualitative effects of pharmacological agents and conditions and thus allow identification of Ca^{2+} transport processes that are important during ischemia. Although the dye is single wavelength, it was demonstrated that the fluorescence changes were not artifacts of cell volume changes by showing that (1) they occurred when cell volume was not changing, during ischemia in glucuronate buffer, and (2) there was no change in fluorescence simply as a result of a volume increase by elevated extracellular K^+ in the absence of extracellular Ca^{2+} (Lipton, 1973; Andrew and MacVicar, 1994; Takahashi et al., 1995). This study identified Ca^{2+} entry via the NMDA receptor and release of Ca^{2+} from the mitochondria via the mitochondrial $2Na^+$–Ca^{2+} exchanger as major contributors to the increase in $[Ca^{2+}]_i$ in neuropil of the hippocampal CA1 region. It is not known whether the same mechanisms apply to somata.

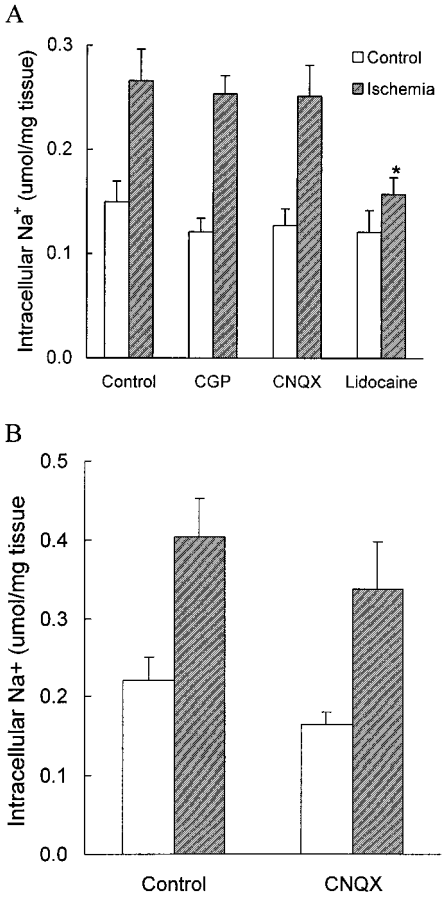

Figure 7. Intracellular Na^+ content after 7.5 min of ischemia in whole hippocampal slices and in CA1 s. radiatum. The measurement of intracellular Na^+ in the slice was as described elsewhere (Kass et al., 1993). After each experiment, slices were washed in ice-cold isotonic sucrose for 10 min to remove extracellular Na^+ and then dried at 80°C overnight. The tissue was weighed and extracted in 0.1N nitric acid overnight, and total Na^+ in the supernatant was measured with a flame photometer. *A*, Intracellular Na^+ content in the whole slice. CGP37157 (*CGP*; 10 μM; $n = 4$), CNQX (20 μM; $n = 4$), and lidocaine (50 μM; $n = 6$) were added to the normal buffer 30 min before ischemia ($^*p < 0.05$, compared with control ischemia). *B*, Effect of CNQX on intracellular Na^+ content at the end of 7.5 min of ischemia in the CA1 s. radiatum. CNQX showed no significant effect on intracellular Na^+ content in this region ($n = 7$).

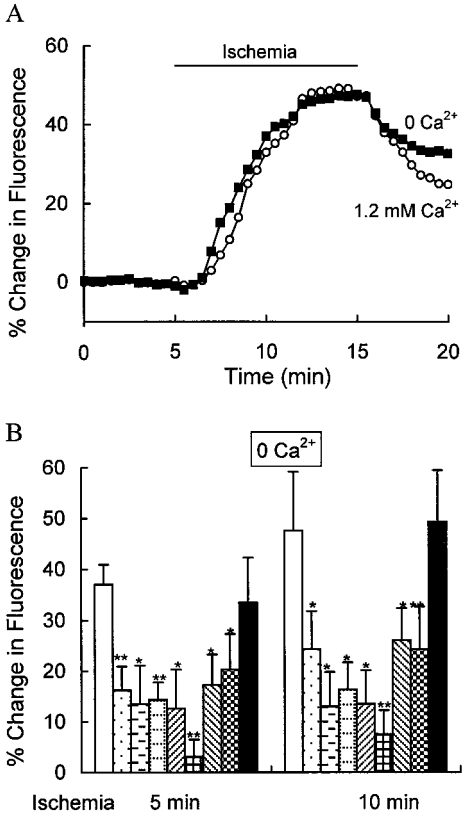

Figure 8. $[Ca^{2+}]_i$ during ischemia in Ca^{2+}-free medium: effects of mitochondrial exchange blocker and Na^+ entry blockers. *A*, The increase in fluorescence in Ca^{2+}-free medium was comparable with that in normal Ca^{2+} buffer. The *horizontal bar* represents the duration of ischemia (10 min). *B*, The fluorescence increase during ischemia in Ca^{2+}-free medium was suppressed by the same manipulations that were effective in normal Ca^{2+} buffer except that MK-801 did not show any effect in Ca^{2+}-free medium. Drug treatments were as follows (from *left* to *right*): control, CGP37157, CNQX, GYKI52466, lidocaine, CNQX + lidocaine, low Na^+, low Na^+ + CGP37157, and MK-801, respectively ($^*p < 0.05$ and $^{**}p < 0.001$, compared with each control value; $n = 5$–8).

Ca^{2+} influx pathways that contribute to the elevation of $[Ca^{2+}]_i$ during ischemia

MK-801 attenuated the early increase in $[Ca^{2+}]_i$, reached after 5 min of ischemia, by 50% but failed to affect the increase in $[Ca^{2+}]_i$ after 10 min of ischemia. This result is consistent with other *in vitro* and *in vivo* studies showing that early Ca^{2+} entry and increased cytosolic Ca^{2+} are dependent on NMDA receptors but that later entry and cytosolic Ca^{2+} are independent of these receptors (Benveniste et al., 1988; Silver and Erecinska, 1990; Lobner and Lipton, 1993).

The small contribution made by NMDA receptors to the overall Ca^{2+} increase is consistent with the minimal role played by these receptors in global ischemic damage (Buchan et al., 1991; Zhang et al., 1997). This probably results from the desensitization of the receptors because of the large fall in ATP (Lobner and Lipton, 1993). NMDA receptors are more important in focal ischemic damage (Buchan et al., 1992), probably because the receptors do not desensitize at the much higher levels of ATP maintained in the focal penumbra (Folbergrova et al., 1992).

There were no other apparent extracellular Ca^{2+} sources that significantly contributed to the increase in $[Ca^{2+}]_i$ during ischemia.

Role of mitochondria in the $[Ca^{2+}]_i$ increase during ischemia

Most of the data are consistent with Na^+ entry during ischemia activating Ca^{2+} release from mitochondria via the inner membrane $2Na^+$–Ca^{2+} exchanger. This process seems to be responsible for approximately one-half of the fluorescence increase. CNQX, lidocaine, and low Na^+ buffer each inhibited the sustained rise in $[Ca^{2+}]_i$ by approximately the same amount as did CGP37157 (50%), and the effects of the Na^+ entry-blocking paradigms were not additive with the effects of CGP37157. Previously published studies (Cox et al., 1993; Baron and Thayer, 1997; White and Reynolds, 1997), along with the drug's lack of effect on Na^+ entry through the plasma membrane and its efficacy in 0-Ca^{2+} buffer, argue very strongly that CGP37157 is acting by blocking the mitochondrial $2Na^+$–Ca^{2+} exchanger.

Although lidocaine did inhibit the measured increase in cytosolic Na^+ during ischemia, CNQX did not, even when measured in neuropil. Despite this, it is still likely that CNQX is acting by blocking Na^+ entry and the resultant $2Na^+$–Ca^{2+} exchange at

the mitochondrial membrane. CGP37157 did not have any additional effect in its presence; CNQX blocked the $[Ca^{2+}]_i$ increase during ischemia in 0-Ca^{2+} buffer, showing that it was not acting by blocking Ca^{2+} fluxes through non-NMDA receptors; and finally, a structurally dissimilar AMPA receptor blocker, GYKI52466, had the same effect as did CNQX. The later argues strongly that CNQX is not acting in an unforeseen way, for example, by a direct action on mitochondrial Ca^{2+} release. The inability to see the effect of CNQX on Na^+ uptake in the whole neuropil is very probably because the AMPA/kainate receptors are strongly localized around dendritic spines on CA1 pyramidal cells (Baude et al., 1995) so that the rise in Na^+ is very localized.

In normal Ca^{2+} buffer, there is net uptake of Ca^{2+} into mitochondria during ischemia despite the reduced oxidative metabolism (Taylor et al., 1999). This net uptake should be greater when the mitochondrial $2Na^+$–Ca^{2+} exchanger is blocked, leading to a smaller increase in $[Ca^{2+}]_i$, as occurs after exposure of cell cultures to glutamate (White and Reynolds, 1997). In 0-Ca^{2+} buffer, there is net release of Ca^{2+} from mitochondria because there is no Ca^{2+} influx. In this case blockade of the $2Na^+$–Ca^{2+} exchange reduces the increase in $[Ca^{2+}]_i$ by reducing this net release.

Multiple roles of Na^+ influx in causing the $[Ca^{2+}]_i$ increase: effects of lidocaine

Lidocaine's effects are almost undoubtedly caused by blockade of persistent or noninactivating Na^+ channels (Taylor, 1993; Lipowski et al., 1996). Its efficacy in the absence of extracellular Ca^{2+} shows that it is not acting by blocking Ca^{2+} currents.

The blockade reduces glutamate release during ischemia (Lekieffre and Meldrum, 1993; Taylor et al., 1995). This is likely to account for lidocaine's inhibition of the very early increase in $[Ca^{2+}]_i$, which is caused by NMDA receptor activation. It also probably explains lidocaine's inhibition of the steady-state increase in $[Ca^{2+}]_i$ that is also blocked by CNQX. Thus release of glutamate (Lobner and Lipton, 1990), primarily by reversal of the Na^+–glutamate cotransporter (Roettger and Lipton, 1996), is a major trigger for the Na^+ entry into pyramidal cell dendrites and subsequent Ca^{2+} release from mitochondria.

Lidocaine plus CNQX reduced the $[Ca^{2+}]_i$ rise much more than did either drug alone; this was a much larger inhibition than was the effect of CGP37157. A likely explanation is that the combined drug treatment lowers Na^+ entry enough to effectively decrease the activation of the sodium pump that normally results from the Na^+ entry. By slowing the loss of ATP, this would allow enhanced clearance of cytosolic Ca^{2+}. The amount of Na^+ entry is indeed a strong determinant of the rate of ATP fall during *in vitro* ischemia or anoxia (Lobner and Lipton, 1993; Fried et al., 1995).

Thus, entering Na^+ plays three major roles in the increase of $[Ca^{2+}]_i$. By activating glutamate release, it activates both NMDA and AMPA/kainate receptors. Also, it enhances the fall in ATP, reducing the clearance of cytosolic Ca^{2+} during ischemia.

Alternative effects of CGP37157

The present data strongly suggest that CGP37157 attenuates the $[Ca^{2+}]_i$ elevation during ischemia by inhibiting the mitochondrial $2Na^+$–Ca^{2+} exchanger in the dendrites, because the effect of CGP37157 is occluded by CNQX. It does not appear to act by blocking glutamate release because it did not affect the very early ischemic increase in $[Ca^{2+}]_i$ that is mainly ascribed to NMDA receptor activation and that is blocked by lidocaine. Furthermore, CGP37157 is unlikely to act by attenuating the fall in ATP during ischemia. That fall is accentuated by increased cell Na^+, but CGP37157 did not block Na^+ influx.

Localization of $[Ca^{2+}]_i$ changes

The major contribution to the optical signal is very probably from dendrites, which occupy 53% of the observed area. Presynaptic elements occupy 33% (see Materials and Methods). Because NMDA and AMPA receptors are postsynaptic (Baude et al., 1995), the portion of the $[Ca^{2+}]_i$ increase that they mediate is almost certainly in dendrites. This includes the early rise in Ca^{2+} as well as the portion of the sustained increase that resulted from activation of the $2Na^+$–Ca^{2+} exchanger, because that was blocked by CNQX. The location and origin of the other 50% of the fluorescence increase, which was primarily prevented when lidocaine and CNQX were combined, are unaccounted for at present.

Relationship of calcium changes to ischemic damage

This study highlights the importance of Na^+ entry in the elevation of cytosolic Ca^{2+} during ischemia. There is strong evidence that reducing Na^+ entry with lidocaine or other Na^+ channel blockers is protective in ischemia (Fujitani et al., 1994; Graham et al., 1994; Weber and Taylor, 1994). The present study establishes that the mitochondrial $2Na^+$–Ca^{2+} exchange system provides a major link between such Na^+ entry and increased $[Ca^{2+}]_i$ and, hence, possibly between Na^+ entry and damage. In doing so the study potentially explains the strong protective effects of AMPA/kainate receptor blockers in ischemia (Strasser and Fischer, 1995; Yatsugi et al., 1996; Turski et al., 1998) because Na^+ entry via these receptors is primarily responsible for activation of the $2Na^+$–Ca^{2+} exchanger.

Summary

The data suggest that the early rise in cytosolic Ca^{2+} during *in vitro* ischemia arises from Ca^{2+} influx via NMDA receptors and that the sustained elevation results in part from Na^+ influx via AMPA/kainate receptors and the resultant activation of Ca^{2+} efflux from mitochondria via the $2Na^+$–Ca^{2+} exchanger. The source of Ca^{2+} that accounts for ~50% of the total ischemic $[Ca^{2+}]_i$ increase in normal and 0-Ca^{2+} buffer, whose clearance is increased when lidocaine and CNQX are combined, is presently unknown.

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