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Intracellular Calcium Levels and Calcium Fluxes in the CA1 Region of the Rat Hippocampal Slice during *in vitro* Ischemia: Relationship to Electrophysiological Cell Damage

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Five minutes of oxygen and glucose deprivation (termed "*in vitro* ischemia") causes long-term synaptic transmission failure (LTF) in the CA1 region of the rat hippocampal slice. Dependence of LTF on cell calcium was tested by generating graded reductions in cell Ca. There was a strong correlation between the average level of exchangeable cell Ca in CA1 during ischemia, and the extent of LTF.

In standard buffer, exchangeable cell Ca in CA1 increased by 35% after 3 min of ischemia and remained elevated for the entire 5 min of ischemia. Unidirectional Ca influx increased by 35% during the first 2.5 min of ischemia and remained at that level for the next 2.5 min. There were no changes in unidirectional Ca efflux during this period. Thus, the accumulation results from increased influx of Ca.

Ca influx during the first 2.5 min of ischemia depended entirely on NMDA channels; it was completely blocked by the noncompetitive NMDA receptor antagonist MK-801. However MK-801 had no effect during the second 2.5 min. This inactivation of NMDA-mediated influx during ischemia appears to result from dephosphorylation. Okadaic acid increased Ca influx during the second 2.5 min of ischemia and this increase was blocked by MK-801. The ischemia-induced Ca influx during the second 2.5 min of ischemia was attenuated 25% by nifedipine (50 μM) and an additional 35% by the Na/Ca exchange inhibitor benzamil (100 μM). The AMPA/kainate antagonist DNQX had no effect on the Ca influx.

Antagonists were used to relate Ca influx to LTF. Blockade of enhanced Ca entry during ischemia in standard buffer (2.4 mM Ca) had no effect on LTF, consistent with total cell Ca prior to ischemia being adequate to cause complete LTF. However, MK-801 strongly protected against LTF when the buffer contained 1.2 mM Ca, a more physiological level. MK-801 combined with DNQX prevented transmission damage in standard buffer. Thus, AMPA/kainate receptor activation contributes to ischemic damage, although not by enhancing Ca entry.

[Key words: calcium channels, desensitization, glutamate, hippocampus, ischemia, NMDA, protein phosphatase, sodium/calcium exchange, ATP, anoxia]

Quite small (< 1 μM) increases in cytosolic Ca appear to lead to severe damage and cell death in many cell types (Nicotera et al., 1986; Orrenius et al., 1989; Michaels and Rothman, 1990; Smith et al., 1991; Randall and Thayer, 1992), lending support to the postulated role of increases in cytosolic Ca in anoxic/ischemic brain damage (Siesjo, 1988). There are numerous descriptions of increases in whole-cell Ca (Siemkowicz and Hansen, 1981; Kass and Lipton, 1986; Benveniste et al., 1988) and free cytosolic Ca (Silver and Erecinska, 1990; Dubinsky and Rothman, 1991; Uematsu et al., 1991; Kadoyo et al., 1992) during cerebral ischemia. Although there are no quantitative demonstrations that the measured increases are themselves adequate to be toxic, there are results that suggest the importance of Ca in toxicity. Removal of extracellular Ca during ischemia, or blockade of the ischemic Ca increase using pharmacological agents, ameliorates ischemic damage in some systems (Kass and Lipton, 1982, 1986; Roberts and Sick, 1988; Amagasa et al., 1990; Uematsu et al., 1991). Furthermore, inhibition of the Ca-activated protease calpain protects against ischemic damage in certain cases (Arai et al., 1990; Lee et al., 1991).

There are several possible mechanisms for the increase in intracellular Ca. They include inhibition of the Ca-ATPase at the plasmalemma, opening of transmitter or voltage-dependent calcium channels, and a net increase in Ca influx via the Na/Ca exchanger (Lipton, 1988). All of these mechanisms might well occur during the first few minutes of ischemia as a result of rapid metabolic changes, which include reduced ATP (Albaum et al., 1953; Lipton and Whittingham, 1982), release of glutamate (Benveniste et al., 1984) and other neurotransmitters (Korf et al., 1988; Globus et al., 1989), membrane depolarization (Rader and Lanthorn, 1989), and increased intracellular Na (Kass and Lipton, 1982). The observed cell Ca increase in neuronal cultures appears to result from activation of NMDA receptors (Goldberg et al., 1989) but it is not clear that this is the case in other systems, where effects of anoxia/ischemia are more rapid. Both NMDA receptor–linked and voltage-dependent calcium channel–linked mechanisms appear to contribute to Ca uptake during focal ischemia *in situ* (Uematsu et al., 1991).

The present studies were partially designed to examine more rigorously the importance of Ca by determining the quantitative relationship between cell Ca levels and the extent of damage in the rat hippocampal slice transiently deprived of oxygen and

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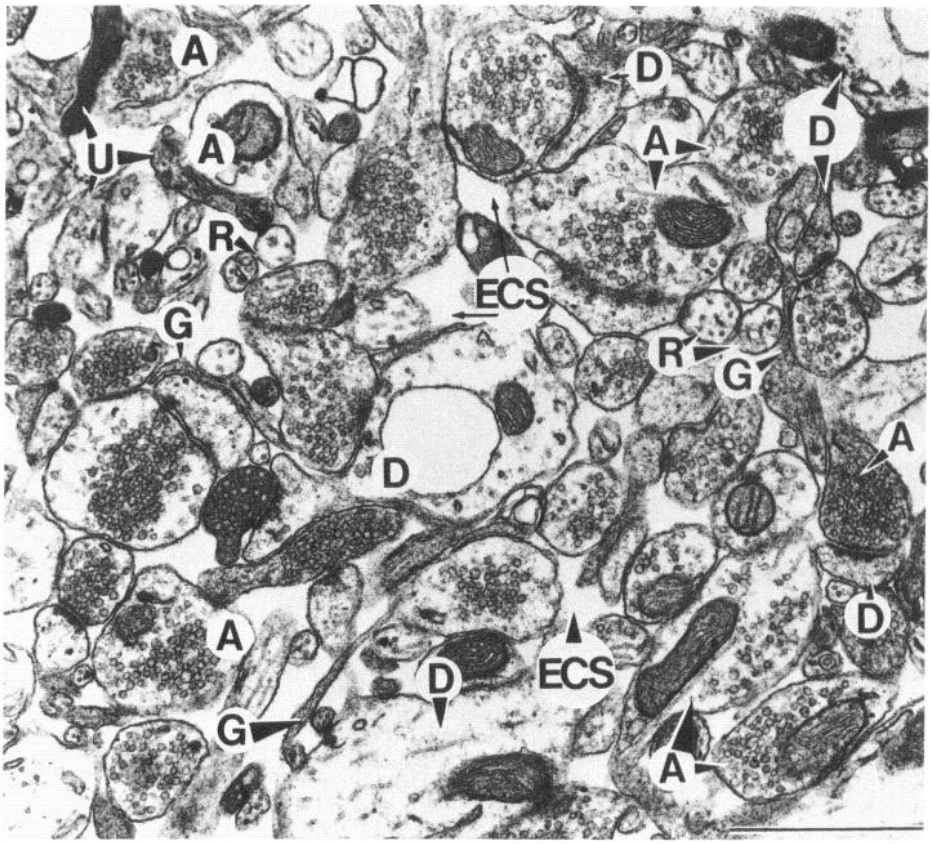

Figure 1. Electron micrograph (25,000×) of CA1 stratum radiatum from slice incubated in standard buffer. *A*, preterminal axons and/or boutons; *D*, dendrites; *ECS*, extracellular space; *G*, presumed glial profiles; *R*, cross sections of either dendritic or axonal elements.

glucose. The mechanisms by which cells in the CA1 hippocampal region accumulate Ca during this *in vitro* ischemia, and the relationship of some of these uptake processes to long-term synaptic transmission failure (LTF) were also determined.

Materials and Methods

Tissue preparation and incubation. Some of the methodology has been described elsewhere (Kass and Lipton, 1982). White male Sprague–Dawley rats, 70–100 d old, were decapitated without anesthesia. The cerebral hemispheres were rapidly removed and placed in ice-cold standard buffer (see below for composition). The hippocampi were removed and 425 μm transverse slices were prepared using a McIlwain tissue chopper. Slices were incubated at 33°C for 2–3 hr on nylon bolting cloth platforms, submerged beneath approximately 1 mm of standard buffer, before beginning each experimental procedure. All experimental procedures were carried out at temperatures between 36°C and 37°C. Procedure for preparation of slices was approved by the University of Wisconsin RARC.

Buffers. Standard buffer contained 124 mM NaCl, 3.0 mM KCl, 1.4 mM KH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 4 mM glucose, equilibrated with 95% O_2, 5% CO_2; pH was 7.4. *In vitro* ischemia buffer was as standard buffer except free of exogenous glucose and equilibrated for 20 min with 95% N_2, 5% CO_2. Conditions in the presence of this buffer are termed "ischemic" (Whittingham et al., 1984). Reduced Ca buffers were as standard buffer except $CaCl_2$ was varied to give appropriate Ca concentrations and no substitution of divalent cations was made. 0 Ca/2 mM EGTA buffer was as standard buffer except $CaCl_2$ was omitted and 2 mM EGTA was added.

Electrophysiological responses. Following the 2–3 hr preincubation, slices were submerged on a nylon bolting cloth platform in a small chamber (about 5 ml vol) that was perfused with approximately 100 ml of equilibrated buffer, recirculated at a rate of 60 ml/min. The Schaffer collateral/commissural pathway was activated by bipolar stimulation and recordings were made in the CA1 pyramidal cell layer using a tungsten microelectrode (Lobner and Lipton, 1990). Stimulation was continued during the ischemic period. The amplitude of the population spike was used to quantify the transynaptic response (Kass and Lipton, 1982). In the absence of intervention the population spike height generally varied by less than 10% over 3 hr periods. In experiments using reduced Ca buffers, slices were returned to normal Ca buffer following ischemia to measure recovery of the population spike.

Measurement of total cell calcium in the CA1 region. Slices were maintained on the same nylon bolting cloth platforms used for preincubation. ^{45}Ca, as $CaCl_2$ (2 μCi/ml), was included in the standard buffer, or in some cases in buffers with altered Ca concentrations, for a total of 50 min. The half-time for ^{45}Ca uptake into the CA1 region is approximately 7 min (Kass and Lipton, 1986), so 50 min is adequate to allow essentially complete equilibration of the extracellular ^{45}Ca with the exchangeable cell Ca pool. Exposure to *in vitro* ischemia was carried out in buffer with the same Ca concentration and specific activities or, in some cases, in buffer containing no extracellular Ca or ^{45}Ca. In both these conditions, changes in cell ^{45}Ca must reflect changes in total cell exchangeable Ca. At the end of the experiment slices were washed in ice-cold buffer containing 2 mM $LaCl_3$ for 1 hr to remove extracellular ^{45}Ca (Kass and Lipton, 1986). The slices were then frozen in liquid nitrogen and dried under vacuum for 12–18 hr, and the CA1 region of the lyophilized slices was isolated by microdissection. The dissected region included the pyramidal cell layer, the stratum radiatum, and about half of the stratum lacunosum-moleculare. Dry tissue weights were measured on a microbalance (Cahn) and ranged from 0.05 to 0.10 mg. Tissue was then dissolved in concentrated nitric acid and the radioactivity measured by scintillation counting. Exchangeable tissue Ca was calculated as (tissue ^{45}Ca)/(specific activity of ^{45}Ca in the loading buffer), and was expressed as nmol Ca/mg dry weight of tissue.

Measurements of calcium influx. Unidirectional influx was approximated by exposing the slices to ^{45}Ca for 2.5 min and measuring ^{45}Ca accumulation as described for total cell Ca measurements. Using the brief period of ^{45}Ca uptake provides a measure of Ca influx that is only slightly contaminated by efflux. The monoexponential equation

$$\mathrm{Ca}(t) = \mathrm{Ca}(T)(1 - \exp(-kt)), \quad (1)$$

where $\mathrm{Ca}(t)$ = total Ca taken up at time t, $\mathrm{Ca}(T) = J/k$ = pool size, J = unidirectional influx, and k = efflux rate constant, is able to account for the ^{45}Ca uptake into the CA1 region of the rat hippocampal slice,

with the efflux rate constant of 0.103 min^{-1} (Kass and Lipton, 1986). It can be calculated from Equation 1 that a 50% decrease or a 100% increase in the efflux rate constant would only affect uptake measured during 2.5 min by 9% and 11%, respectively. Thus, in the absence of large changes in efflux, the 2.5 min uptake is a reliable measure of influx.

Measurement of calcium efflux. Slices were equilibrated with ^{45}Ca for 50 min in standard buffer; slices were then transferred into 0 Ca/2 mM EGTA buffer containing no ^{45}Ca for 2 min to remove extracellular ^{45}Ca. Slices were then exposed to 0 Ca/2 mM EGTA for an additional 0, 2.5, or 5 min in normoxic or ischemic buffer. ^{45}Ca in the CA1 region was measured at these three times in different slices.

ATP measurements. Experimental procedures were similar to those for the cell Ca experiments except tissue was frozen immediately after the experiment. The ATP in the CA1 region was extracted as described previously (Lowry et al., 1964) and measured using a luciferin-luciferase–based assay (Lust et al., 1981; Kass and Lipton, 1986).

Measurement of areas occupied by different cellular elements in stratum radiatum of CA1. Semithin sections were prepared for light microscopy and thin sections were prepared for electron microscopy using standard techniques (Feig and Lipton, 1990). Light microsocopy was used to identify the percentage of the stratum radiatum that was occupied by glial and interneuronal cell bodies. Low-power electron micrographs (7500×) were used to measure the percentage of stratum radiatum that was occupied by large longitudinal dendritic profiles (diameter > 1 μm). High-power (25,000×) micrographs (Fig. 1) were used to measure the percentages of the areas occupied by smaller profiles. These included dendrites, presynaptic axons and boutons, cross-sectional profiles that were either pre- or postsynaptic, unidentified elements that included glial profiles, and the extracellular space. Two slices were analyzed in this way. Four micrographs per slice were analyzed at low power and four at high power. The total area per slice analyzed at low power was 7200 μm^2 and the total area per slice analyzed at high power was 280 μm^2. Areas of tissue elements were measured using a computerized tracing system (Jandel).

Chemicals. MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] was obtained from Merck (Rahway, NJ); DNQX (6,7-dinitroquinoxaline-2,3-dione); benzamil, from Research Biochemicals Incorporated (Natick, MA); okadaic acid, from Molecular Probes (Eugene, OR); and DTG [di(*o*-tolyl)guanidine], from Aldrich (Milwaukee, WI). All other chemicals were obtained from Sigma (St. Louis, MO). All chemicals except calmidazolium, okadaic acid, and benzamil were added to the bathing medium 10 min prior to ischemia and were present during ischemia. Calmidazolium, okadaic acid, and benzamil were added 30 min prior to ischemia and maintained during ischemia.

Data presentation. All error bars represent SEMs. n = number of independent observations. An independent observation was the measurement of an ion concentration, metabolite, or electrophysiological response in one slice. In general, one animal was used for an experiment. For ion and ATP measurements, there were six treatments or incubation times per experiment; four slices were used for each treatment or time. In each experiment there were some slices that were untreated with drugs or ischemia (control normoxia). Values for the other treatments were expressed as percentages of these "control normoxia" values. For electrophysiological measurements up to three different treatments were carried out on slices from one animal. One of these was always a control (no addition) ischemia. Statistical significance of differences between treatments was calculated using Student's t test, unless indicated as analysis of variance with Fisher's least significant difference test.

Results

Relationship between cell calcium and long-term transmission failure

Ischemic cell damage was assessed as long-term loss of the population spike in the CA1 pyramidal cell layer evoked by stimulation of the Schaffer collateral/commissural pathway (Kass and Lipton, 1982). Five minutes of ischemia caused total LTF; there was no recovery of the population spike after 60 min of reoxygenation.

If increased cytosolic Ca is a trigger for LTF, then decreasing the intra- and extracellular sources of Ca during ischemia should protect against the damage. This was tested in two different

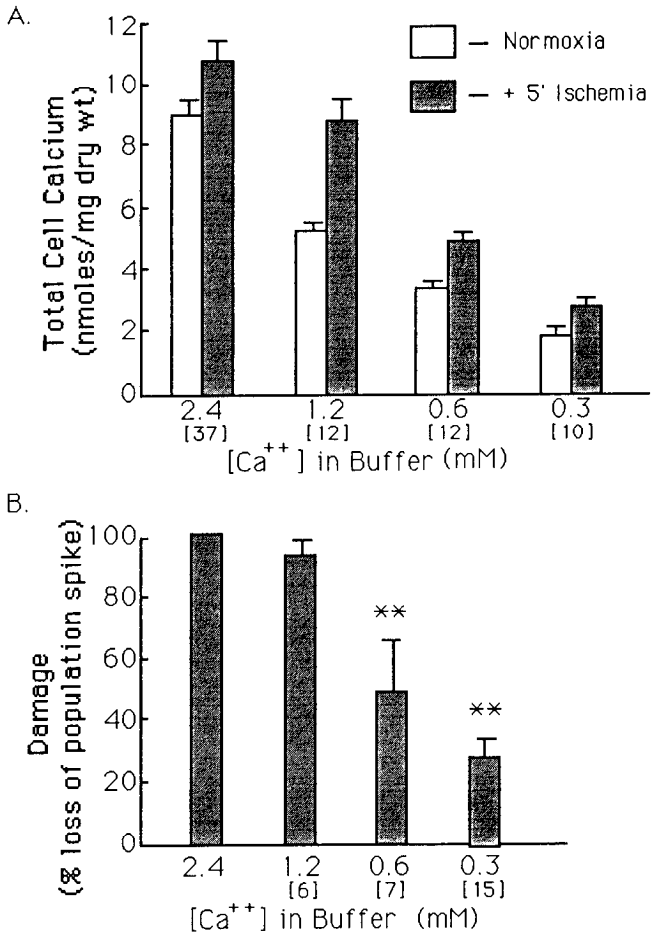

Figure 2. Effects of prolonged exposure to reduced extracellular Ca on transmission damage and cell calcium. Slices were incubated in modified buffers containing the indicated Ca concentrations for 45 min prior to 5 min of ischemia and during the ischemia. In electrophysiological experiments slices were returned to normal Ca buffer 5 min after the ischemia in order to allow measurement of recovery. *A*, Cell calcium measured after 50 min of exposure to ^{45}Ca in different extracellular Ca buffers. *Open bars*, normoxic for entire 50 min; *solid bars*, final 5 min ischemic. *B*, Effect on population spike. Percentage damage = $(1 - P/P_0) \times 100$, where P_0 = population spike amplitude prior to reducing extracellular Ca, and P = population spike amplitude measured 60 min after 5 min of ischemia. **, Significantly different from 100% damage, $p < 0.01$. Numbers in brackets = n.

experimental paradigms. In the first, extracellular Ca was reduced to different levels for 45 min prior to ischemia as well as during the 5 min ischemic period. There were marked reductions in intracellular Ca prior to ischemia and intracellular Ca increased from this value during *in vitro* ischemia (Fig. 2*A*). Lowering extracellular Ca to a physiological level, 1.2 mM, reduced cell Ca by about 40%, but did not significantly attenuate LTF (Fig. 2*B*). However, more profound reductions in intracellular Ca were associated with significant attenuation of LTF (Fig. 2*B*).

In another paradigm, tissue was exposed to nominally Ca-free (0 Ca) buffer for different durations prior to the 5 min of ischemia, and during the ischemia. Figure 3*A* shows that cell Ca declined as the time of exposure to 0 Ca increased, and that in all cases it decreased further during the 5 min ischemic interval. Removing extracellular Ca only during the 5 min of ischemia gave slight protection against LTF (0′ in Fig. 3*B*).

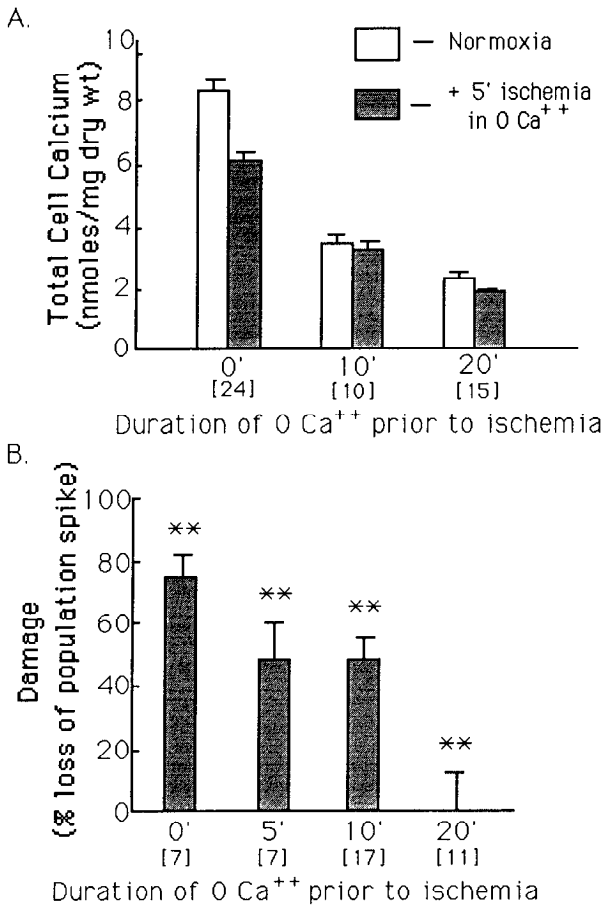

Figure 3. Effects of 0 Ca incubation on transmission damage and cell calcium. Slices were incubated in 0 Ca buffer prior to ischemia for the periods indicated. For electrophysiological studies slices were returned to normal-Ca buffer 5 min after ischemia. *A,* Slices were incubated in standard buffer with ^{45}Ca for 50 min followed by varying periods in 0 Ca buffer containing no ^{45}Ca. *Open bars,* slices removed after duration shown in normoxic buffer; *solid bars,* slices in ischemic buffer with 0 Ca for an additional 5 min. *B,* Damage to synaptic transmission measured as in Figure 2.

However, when slices were incubated in 0 Ca buffer for periods prior to the ischemia, there was far better protection. Twenty minutes of preincubation in 0 Ca buffer provided full protection against LTF; there was no measurable damage. Figure 4 is a summary of the two studies described in Figures 2 and 3, and also includes a data point showing the effect of exposing slices to 0 Ca buffer containing 2 mM EGTA for 5 min prior to and during ischemia. There is a good correlation ($r = 0.927$) between the extent of LTF and the mean level of cell Ca during the ischemic period over the entire range of cell Ca, regardless of whether there was influx (lowered Ca) or efflux (nominally 0 Ca) of Ca during the ischemic interval.

Effects of lowered Ca on ATP levels

A possible explanation for the protective effect of lowered Ca is that it decreased the rate at which ATP fell during ischemia. Figure 5 shows this was not the case. The two Ca incubation paradigms that most strongly protected the slice against LTF actually increased the rate at which ATP fell during *in vitro* ischemia in the CA1 region. This acceleration appears to result

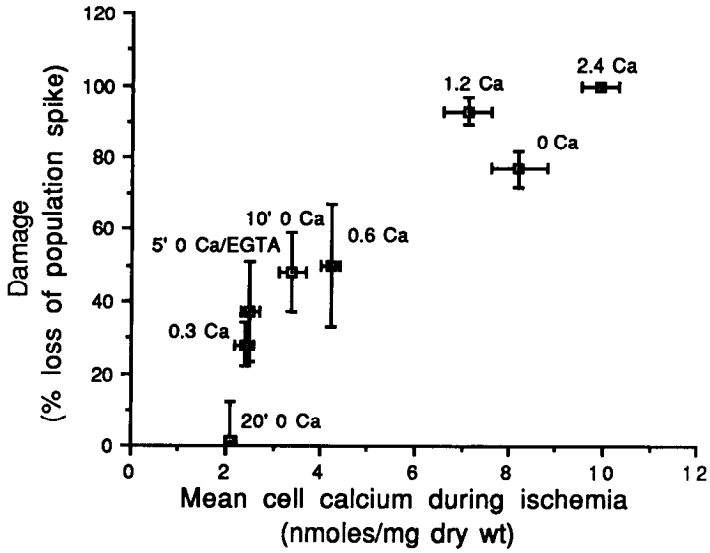

Figure 4. Relationship between transmission damage and cell calcium. This figure was constructed from data in Figures 2 and 3 with addition of one study (*5' 0 Ca/EGTA*). In that study slices were exposed to 0 Ca buffer containing 2 mM EGTA for 5 min prior to ischemia, during, and for 5 min after ischemia. Mean cell calcium during ischemia is the average between the cell calcium prior to ischemia and that at the end of ischemia. The linear correlation coefficient for the relationship between LTF and cell calcium is 0.927.

from enhanced Na entry during the ischemia (D. Lobner and P. Lipton, unpublished observations). The 0 Ca paradigm also does not appear to be protective by enhancing ATP recovery after ischemia. ATP levels 10 min following 5 min of ischemia in normal Ca buffer returned to 73 ± 3% of control; in the 0 Ca paradigm, ATP levels had recovered to 66 ± 3% of control.

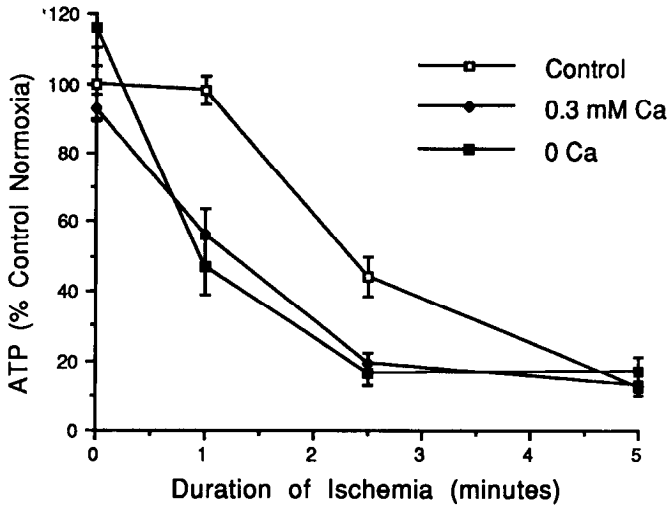

Figure 5. Effects of 0.3 mM Ca and 0 Ca on ATP levels in the CA1 region of the rat hippocampal slice during ischemia. For *0.3 mM Ca,* slices were exposed to 0.3 mM Ca for 45 min prior to and during ischemia. For *0 Ca,* slices were exposed to 0 Ca buffer for 20 min prior to the ischemic period and also during ischemia. ATP levels are expressed as percentage of levels in standard buffer prior to ischemia (*Control* normoxia). ATP in control normoxia slices = 5.2 ± 0.5 nmol/mg dry wt; n = 13–26 for control, 7–12 for 0.3 mM Ca, 8–12 for 0 Ca. Values for 0 Ca and 0.3 mM Ca at 1 and 2.5 min of ischemia are significantly different from control ischemia, $p < 0.01$.

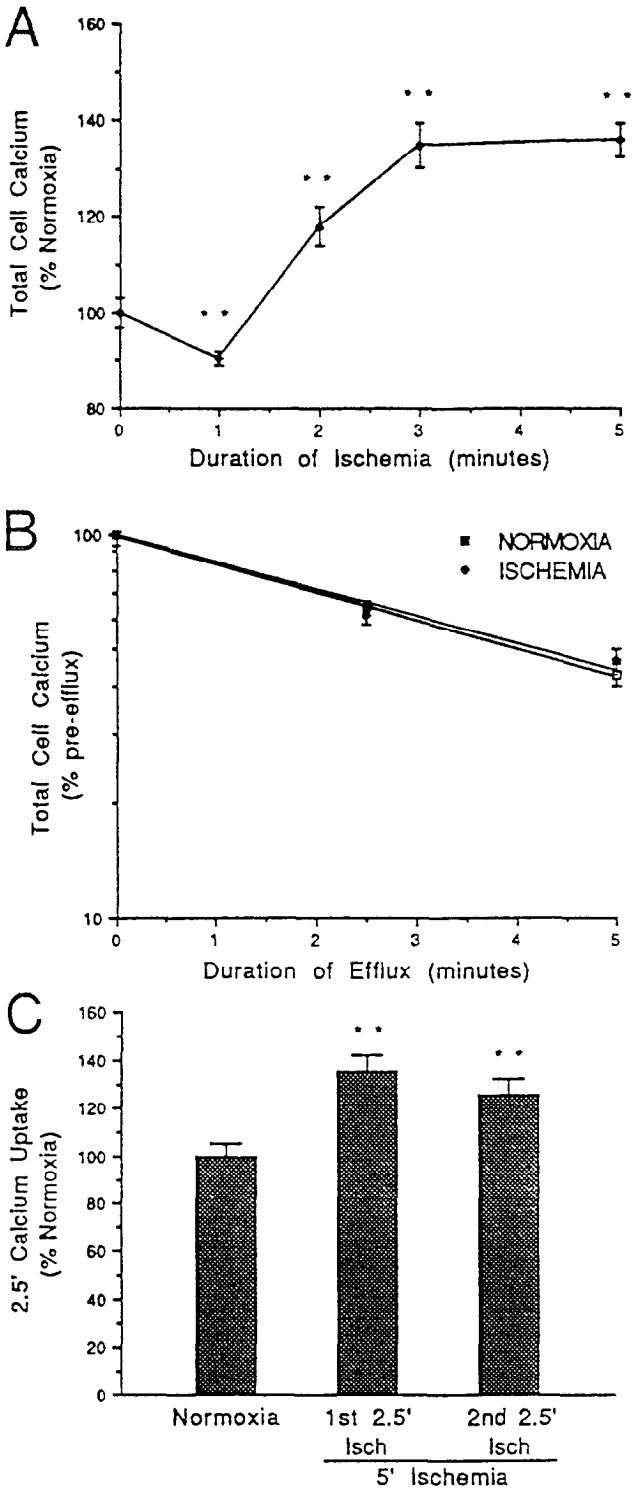

Figure 6. Total calcium accumulation and unidirectional fluxes during *in vitro* ischemia *A*, Total cell calcium. Cell calcium content is expressed as percentage of levels in standard buffer prior to ischemia (control normoxia). Calcium in control normoxia = 9.0 ± 0.6 nmol/mg dry wt. In each experiment the slices were exposed to ^{45}Ca for a total of 50 min; the last 0–5 min were ischemic. n = 20–51 for different time points. Ischemia values are significantly different from normoxia for all time points ($p < 0.01$). *B*, Ca efflux. Slices were loaded with ^{45}Ca for 50 min and then placed in 0 Ca/2 mM EGTA buffer for 2 min to remove extracellular ^{45}Ca. Slices were then exposed to 0 Ca/2 mM EGTA in either normoxic or ischemic buffer for the time shown on the abscissa, and then analyzed for ^{45}Ca. Cell calcium prior to efflux = 7.1 ± 0.3 nmol/mg dry wt. n = 20 for all points. There is no significant difference between the efflux rate constant during normoxia and ischemia; k = 0.17 min^{-1} for both conditions. *C*, Ca influx. Slices were exposed to ^{45}Ca for 2.5 min either during normoxia or during the first or second periods of *in vitro* ischemia. n = 18. **, Significantly different from normoxia, $p < 0.01$.

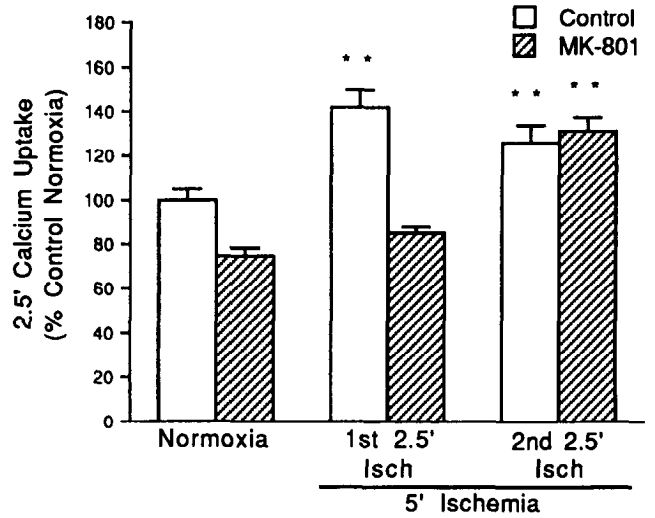

Figure 7. Effect of MK-801 on ^{45}Ca uptake during ischemia. Slices were exposed to ^{45}Ca for 2.5 min in normoxia, at the onset of ischemia (*1st 2.5'*), or after 2.5 min of ischemia (*2nd 2.5'*). The NMDA antagonist MK-801 blocked the increased calcium influx during the first 2.5 min of ischemia, but did not block the increase during the second 2.5 min of ischemia. The drug reduced uptake in normoxia. Uptake in control normoxia, 4.3 ± 0.2 nmol/mg dry wt/2.5 min; n = 18. **, Significantly different from normoxia for the same treatment (control or drug), $p < 0.01$.

Kinetics of calcium changes during 5 min of in vitro *ischemia*

The importance of cell Ca in transmission damage led to an investigation of the mechanisms by which this parameter increased during *in vitro* ischemia.

There was a 10% decrease in total cell Ca after 1 min of ischemia, followed by a rapid increase over the next 2 min. Elevated levels of cell Ca were then maintained for the duration of the 5 min ischemic period (Fig. 6*A*). The early decrease is probably due to release of Ca from intracellular stores into the cytosol, with subsequent stimulation of Ca efflux processes.

Unidirectional calcium fluxes during 5 min of in vitro *ischemia*

The increase in cell Ca could result from increased Ca influx, decreased Ca efflux, or a combination of both. Figure 6*B* shows that ischemia had no effect on ^{45}Ca efflux into a Ca-free EGTA-fortified buffer. The rate constant in both cases was 0.17 min^{-1}. Figure 6*C* shows that ischemia increased the influx of ^{45}Ca during both the first and second halves of the 5 min of ischemia. The increases were similar during the two periods and were both similar to the net uptake occurring during the ischemia (about 35%).

These results strongly suggest that the Ca accumulation during *in vitro* ischemia can be accounted for by an activation of influx processes. The nature of these processes was investigated.

Effects of MK-801 on Ca influx

As shown in Figure 7, 10 μM MK-801, a noncompetitive NMDA receptor antagonist (Wong et al., 1988), reduced normoxic influx

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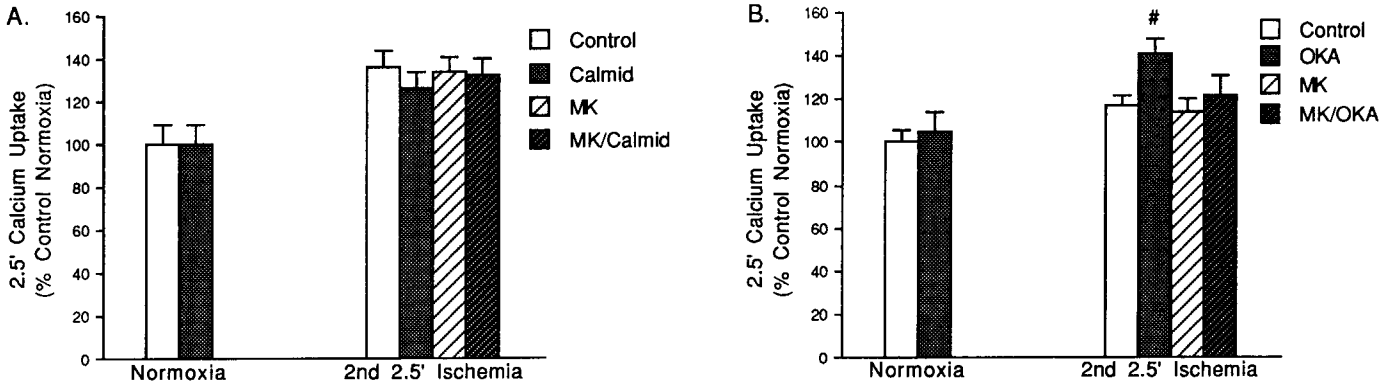

Figure 8. Effects of calmidazolium and okadaic acid on ^{45}Ca uptake during the second 2.5 min of ischemia. Slices were treated as described for Figure 7. *A*, Calmidazolium at 5 μM did not affect normoxic or ischemic calcium uptake. *B*, Okadaic acid at 0.5 μM increased calcium uptake during the second 2.5 min of ischemia; this increase was blocked by MK-801. $n = 12$. *Calmid*, 5 μM calmidazolium; *MK*, MK-801; *OKA*, 0.5 μM okadaic acid. #, Significantly different from control ischemia, $p < 0.05$, by ANOVA and Fisher's least significant difference test.

by approximately 25%, indicating that a component of resting Ca influx is via NMDA channels. This reduction cannot be a result of decreased diffusion through the intercellular spaces, as an identical lowering of *total* cell Ca occurred (data not shown). MK-801 also completely blocked the increased influx during the first 2.5 min but had no effect on the influx during the second 2.5 min of ischemia. Thus, early Ca entry appears to rely completely on flux through NMDA receptor–linked channels. However, this mechanism appears to inactivate after a short period of ischemia, as it makes no contribution to influx during the second 2.5 min period. The absence of any NMDA-mediated Ca influx during this late period shows that even the NMDA receptor–mediated influx occurring in normoxic conditions is blocked.

Inactivation of NMDA-mediated calcium influx

The apparent elimination of Ca flux through the NMDA channel during the second 2.5 min of ischemia may have resulted from dephosphorylation of the receptor-channel complex (MacDonald et al., 1989) following the rapid fall in ATP levels. This was tested using inhibitors of protein phosphatases. Calmidazolium, which blocks calcium/calmodulin activation of calcineurin (Gietzen et al., 1981), did not affect Ca influx during the second 2.5 min of ischemia (Fig. 8*A*). However, okadaic acid, which blocks the constitutively active phosphatases 1 and 2a (Cohen et al., 1990), enhanced influx during the second 2.5 min of ischemia. Furthermore this enhancement was completely abolished by MK-801 (Fig. 8*B*). This indicates that dephosphorylation of NMDA-sensitive channels accounts for the abolition of Ca entry through these channels during the *in vitro* ischemia.

Components of late calcium influx

The change of influx during the second 2.5 min of ischemia, as compared with control conditions, included the elimination of the normoxic NMDA-mediated Ca influx as well as any new fluxes caused by ischemia. In order to isolate the latter, 10 μM MK-801 was included in the buffer throughout these experiments.

Figure 9 shows that the AMPA/kainate receptor antagonist DNQX (100 μM) did not block ischemia-induced influx, nor did the α1-antagonist prazosin at a concentration of 10 μM (data not shown).

The voltage-dependent Ca channel antagonist nifedipine (50 μM) reduced the Ca influx by 25%, and benzamil (100 μM), an inhibitor of the Na/Ca exchange carrier, blocked an additional 35% (Fig. 10). Benzamil was added in the presence of nifedipine to ensure that its effect was not due to blockade of L-type voltage-dependent calcium channels (Kleyman and Cragoe, 1988). Approximately 40% of the late ischemic increase was unaffected by these inhibitors.

The inhibitory effect of benzamil did not differentiate between whether *in vitro* ischemia activated the Ca/Na exchange or Ca/Ca exchange mode of the Na/Ca exchanger. Figure 11 shows results of a study designed to help resolve this. Figure 11*A* shows that, during normoxia, 100 μM benzamil completely blocked additional Ca influx caused by removal of sodium but had no effect on basal Ca influx. This indicates, first, that in the resting state the Ca influx via the Na/Ca exchanger is negligible. Second, it demonstrates that 100 μM benzamil does completely block Ca influx via the exchanger, when the latter is active. Figure 11*B* shows that the normal increase in Ca influx during the second 2.5 min of ischemia was reduced approximately 40% when NaCl was removed from the bathing medium during the experiment (compare the difference between the dark bars with the difference between the light bars). The size of the reduction

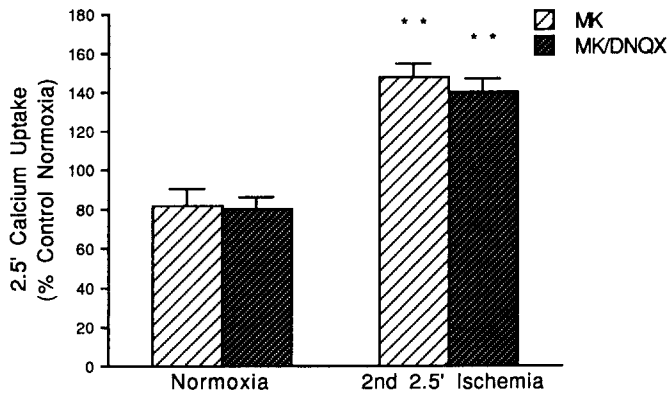

Figure 9. Effects of an AMPA/kainate receptor antagonist on ^{45}Ca uptake during the second 2.5 min of ischemia. Slices were treated as for Figure 7. DNQX was tested in the presence of 10 μM MK-801 (*MK*). DNQX at 100 μM did not affect calcium uptake during the second 2.5 min of ischemia. $n = 12$ for DNQX experiments. **, Significantly different from normoxia for the same treatment, $p < 0.01$.

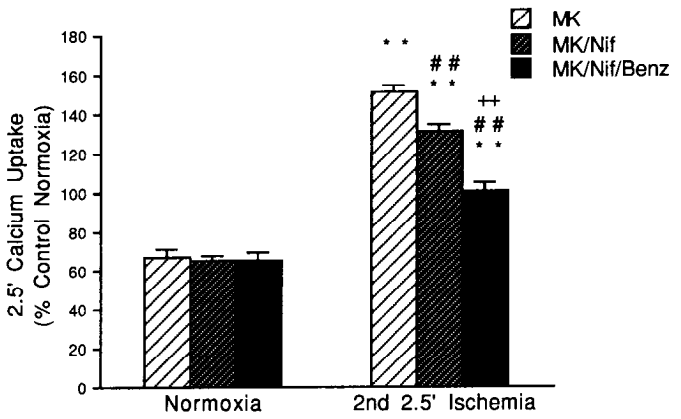

Figure 10. Effects of nifedipine and benzamil on ^{45}Ca uptake during the second 2.5 min of ischemia. Slices were treated as for Figure 7. Antagonists were tested in the presence of MK-801 (*MK*). *Nif*, 50 μM nifedipine; *Benz*, 100 μM benzamil. $n = 12$. **, Significantly different from normoxia for the same treatment, $p < 0.01$; ##, significantly different from *MK* ischemia, $p < 0.01$; ++, significantly different from *MK/Nif* ischemia, $p < 0.01$. Significance tested by ANOVA and Fisher's least significant difference test.

was very similar to the size of the reduction caused by benzamil during ischemia in normal buffer (Fig. 10). This similarity suggests that the ischemic Ca influx blocked by benzamil is due to Na entry and resultant Na/Ca exchange flux, and hence represents a net Ca entry.

Relationship of calcium movements to ischemic transmission damage

Five minutes of ischemia caused complete LTF (Fig. 12, CONT). Neither NMDA antagonists (MK-801, CGS-19755) nor nifedipine provided significant protection against damage in spite of their effects on Ca fluxes, nor was protection provided by the combination of MK-801, nifedipine, and benzamil. Thus, combined blockade of the early Ca entry and most of the later Ca entry did not prevent the damage when the slices were incubated in 2.4 mM Ca–containing buffer.

In contrast, *combined* blockade of NMDA-mediated channels and non-NMDA glutamate receptors provided strong protection against LTF (Fig. 12). Either DNQX/glycine, which blocks AMPA/kainate receptors, or DTG, which blocks ischemic glutamate accumulation in the extracellular medium and in this way appears to block activation of AMPA/kainate receptors (Lobner and Lipton, 1990), provided strong protection against LTF, when combined with MK-801. Ketamine (1 mM), which blocks both glutamate accumulation (Lobner and Lipton, 1990) and NMDA channels, protected against LTF, as did 10 mM kynurenic acid, which blocks both the ionotropic glutamate receptor classes (Ganong et al., 1983).

In contrast to its effect in buffer containing 2.4 mM Ca, MK-801 strongly protected against LTF when extracellular Ca was at a more physiological level, 1.2 mM. (Fig. 13).

Discussion

Methodological considerations

Possible effects of extracellular space changes on ^{45}Ca measurements. It is important to assess any artifactual effects of extracellular space changes on the measured ischemic uptake of ^{45}Ca. Cell swelling rapidly decreases the extracellular space during anoxia/ischemia (Harrevald and Ochs, 1956; Boer et al., 1990). This decreases volume fraction and increases tortuosity of the intercellular spaces and so should slow diffusion of Ca into the slice (Nicholson, 1988–89). The magnitude of the effect is hard to predict, but it will clearly reduce the measured Ca uptake. It is improbable that the increased tortuosity will remain during the ice-cold $LaCl_3$ washout, as both sets of slices are in aerated ice-cold buffer for 60 min. If it does it should not affect efflux from the extracellular space during this washout as the half-time for removal from that space is 4 min (Kass and Lipton, 1986) compared with the 60 min during which slices are washed. Thus, the net effect of the cell volume changes should be, if anything, to cause an underestimate of the change in unidirectional influx.

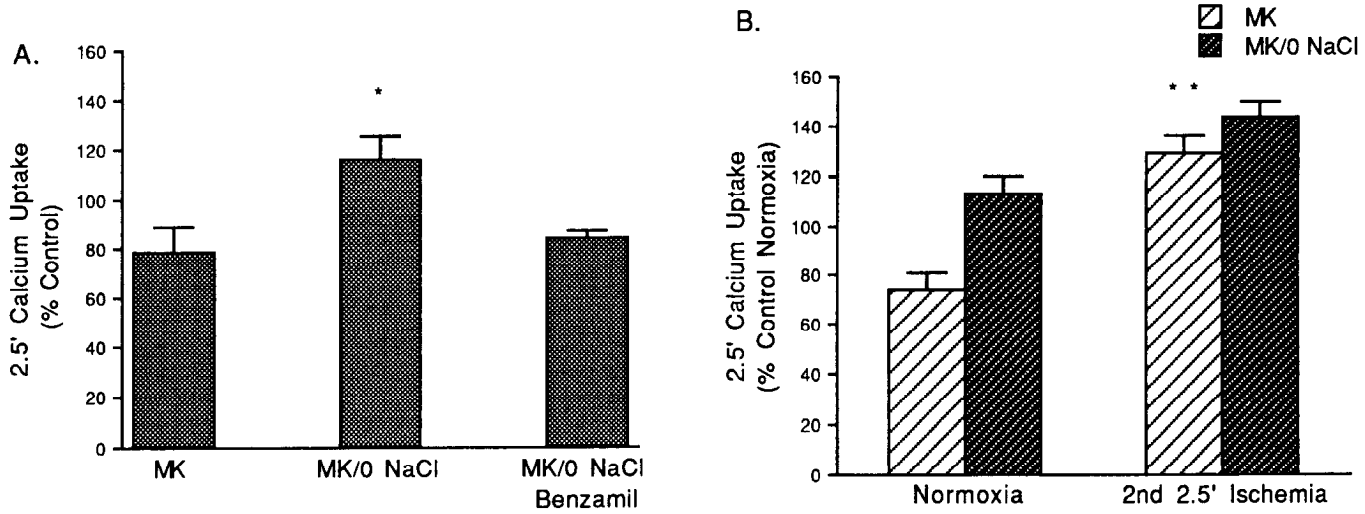

Figure 11. Nature of benzamil-inhibitable Ca influx. Slices were treated as for Figure 7. Experiments were performed in the presence of MK-801. NaCl in the bathing medium was replaced with choline-Cl 10 min prior to and during the 2.5 min ^{45}Ca uptake period. *A*, Benzamil blockade of Ca influx in low-Na buffer. The reduced-Na buffer significantly stimulated calcium uptake (*, $p < 0.05$) and this increase was blocked by 100 μM benzamil. $n = 8$–16. *B*, Effects of low-Na buffer on ^{45}Ca uptake during the second 2.5 min of ischemia. The rise in ^{45}Ca uptake during ischemia was reduced in low-Na buffer. $n = 16$. *, significantly different from normoxia for the same treatment, $p < 0.01$.

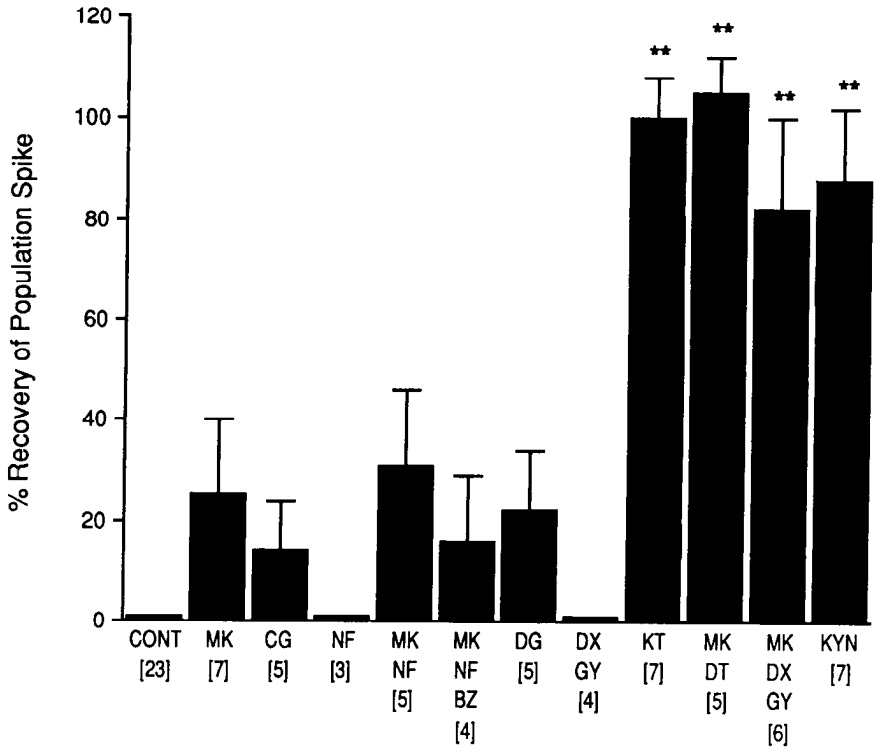

Figure 12. Effect of antagonists on recovery of the population spike after 5 min of ischemia. Recovery is defined as the amplitude of the population spike 60 min after 5 min of ischemia as a percentage of the amplitude prior to the addition of any drug. *CONT*, no drug; *MK*, 10 μM MK-801; *CG*, 100 μM CGS-19755; *NF*, 50 μM nifedipine; *BZ*, 100 μM benzamil; *DG*, 25 μM DTG; *DX*, 100 μM DNQX; *GY*, 100 μM glycine; *KT*, 1 mM ketamine; *KYN*, 10 mM kynurenic acid. **, significant protection, $p < 0.01$. Some of the results have been published previously (Lobner and Lipton, 1990) but are included for clarity.

Localizations of ^{45}Ca. Implicit in the interpretation of the results is that the measured calcium changes reflect changes in neuronal compartments that are undergoing damage. These include either or both of the two components of the electrophysiological pathway, the terminals of the Schaffer collaterals, and the dendrites/cell somata of the pyramidal cells.

About 10% of the microdissected region comprised the pyramidal cell layer, which is approximately 90% pyramidal cell somata and dendrites (Sloviter, 1989). There is very little glial tissue, especially in the slice (Feig and Lipton, 1990).

The great majority of the tissue in stratum radiatum comprised dendritic profiles and presynaptic axons and boutons: dendrites (including spines), 47 ± 4%; presynaptic fibers and boutons, 31 ± 3%; cross-sectional profiles of either small axons or dendrites, 9 ± 2%; other (including glial profiles and unidentified fragments), 14 ± 3%. Glial cell bodies occupy less than 1% of the region (0.09 ± 0.03%) The extracellular space was 10 ± 5% of the tissue (see Materials and Methods). Although their origin is not precisely known, the great majority of the dendrites are part of CA1 pyramidal cells (Schlander and Frotscher, 1986; Feig and Lipton, 1993), and the presynaptic elements should be almost completely associated with Schaffer collateral or commissural fibers, as these make up the overwhelming preponderance of fibers in this region (Ishizuka et al., 1990). Thus, tissue in stratum radiatum is largely associated with elements that were studied electrophysiologically.

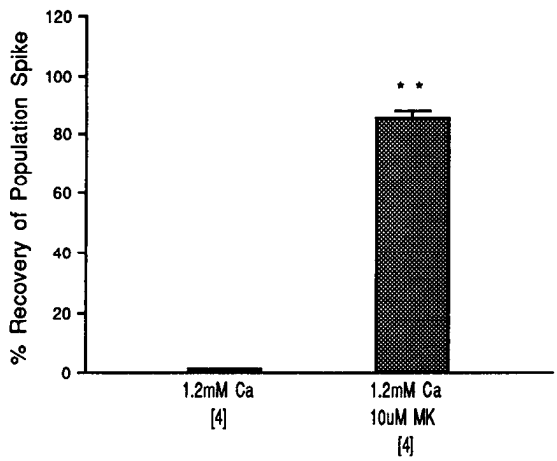

Figure 13. Effects of MK-801 on recovery of the population spike in 1.2 mM Ca buffer. The standard buffer was changed to 1.2 mM Ca buffer 45 min prior to ischemia and during ischemia. Standard buffer was reinstated for the recovery period. **, significant protection, $p < 0.01$.

Stratum lacunosum-moleculare comprised approximately 20% of the dissected region. It includes distal dendrites from the CA1 pyramidal cells but is largely made up of terminals and interneurons that were not studied electrophysiologically. It was included because the stratum lacunosum-moleculare/stratum radiatum border is difficult to identify with certainty in lyophilized tissue.

The above analysis shows that approximately 65% of the tissue in the microdissected sample is composed of cellular elements that were studied electrophysiologically. This includes ascribing 10% of dendrites and presynaptic elements in stratum radiatum to cells that were not part of the electrophysiological pathway being studied. Thus, the precise extent to which the measured cell Ca represents changes in tissue that was damaged cannot be calculated. However, the potentially damaged elements do represent about two-thirds of the analyzed tissue, so unless they are far less sensitive to the manipulations in these studies than are other tissue elements, the measured changes should largely reflect changes in them. In the analysis of the data it is assumed that the measured percentage changes in Ca, and ATP, do represent percentage changes in damaged elements.

Calcium and long-term transmission failure

A principle aim of this study was to determine whether there is a positive relationship between cell Ca levels during ischemia, and the extent of ischemic damage. Such a relationship must be true over some range if cell Ca is a cause of damage.

Intracellular Ca was varied in two different ways and in both cases there was a positive monotonic relation between the average amount of Ca within the cells and damage. For this 5 min *in vitro* exposure, transmission damage was complete when mean Ca levels during the *in vitro* ischemia were somewhat less than those in resting cells at 2.4 m*M* Ca_o (Fig. 2). At the other extreme, there was zero damage when cell Ca was 20% of the resting value.

The relationship between damage and Ca cannot be explained by Ca accelerating the fall in ATP levels. The rate at which ATP declines during ischemia was actually accelerated in the 0 Ca conditions (Fig. 5). This result, coupled with previous results on precisely the same system, which showed that glutamate release during *in vitro* ischemia is not affected by 20 min of 0 Ca incubation (Lobner and Lipton, 1990), establish quite firmly that a parameter related to total cell Ca acts directly to initiate, or help initiate, ischemic damage to synaptic transmission. This parameter may well be cytosolic Ca, which should reflect total cell Ca as a result of reequilibration of energy-dependent stores across organelle membranes during ischemia (Siesjo, 1988). While the precise quantitative relationship between total cell Ca and cytosolic Ca during ischemia is not known, the fact that organelle accumulation is energy dependent and cytosolic (Ca) dependent (Siesjo and Bengtsson, 1989; Gunter and Pfeifer, 1990) implies that cytosolic levels during ischemia will increase with both the decreased rate of oxygen utilization and the amount of Ca in organelles and extracellular medium.

The results in 0 Ca buffer demonstrated that some cell damage occurred without any increase in total cell Ca; however, in normal extracellular media there is a net Ca influx during *in vitro* ischemia and this will contribute to damage by elevating total cell Ca.

Calcium efflux during ischemia

There was no measured change in the ^{45}Ca efflux during 5 min of ischemia. Earlier studies had shown a decrease in Ca efflux that was ascribed to inhibition of the Ca-ATPase (Lipton and Lobner, 1990). However, it appears that the reported decrease was an artifact of the experimental procedure, in which ^{45}Ca efflux was measured into a 0 Ca–containing buffer without EGTA. There is significant backflux of Ca from the extracellular space in that situation, illustrated by the much slower efflux rate constant when EGTA was excluded [0.11 min^{-1} without EGTA (Lipton and Lobner, 1990) vs. 0.17 min^{-1} with EGTA in this study]. The increased influx that occurs during *in vitro* ischemia enhances the backflux, and thus leads to an *apparent* reduction in the efflux rate constant during ischemia when the Ca is not chelated by EGTA. The absence of a change in efflux does not necessarily imply that processes contributing to Ca efflux are unaltered by *in vitro* ischemia. It is quite possible that efflux mechanisms at the plasmalemma are inhibited due to low ATP and elevated intracellular Na (DiPolo and Beauge, 1988; Siesjo and Bengtsson, 1989), but that increases in cytosolic Ca due to efflux from organelles maintain plasmalemma efflux rates by increasing the substrate concentrations for these mechanisms.

The lack of any change in efflux led to the conclusion that the basis for the increased Ca uptake during ischemia was an increased unidirectional influx.

NMDA receptor–mediated influx, and its desensitization

The increase in Ca influx during the first 2.5 min of ischemia was completely blocked by 10 μM MK-801, the noncompetitive NMDA antagonist. It is thus probable that the calcium enters through the NMDA-mediated channels, although it is possible that flux through the NMDA channels leads to some Ca flux through other channels. The NMDA-mediated Ca entry is consistent with the rapid release of glutamate during ischemia in this system (Lobner and Lipton, 1990), the early depolarization of the postsynaptic cells (Rader and Lanthorn, 1989), and the well-established Ca conductance of the NMDA-sensitive channels (Dingledine, 1983). There is similar evidence that the early ischemia-induced Ca influx in intact brain is via NMDA channels (Benveniste et al., 1988; Silver and Erecinska, 1990).

In contrast to the first 2.5 min, NMDA antagonists did not affect Ca influx during the second 2.5 min of ischemia, indicating there was no flux through NMDA-sensitive channels. Conditions during this period would favor entry through NMDA channels because the pyramidal cells are clearly depolarized (Rader and Lanthorn, 1989). Thus, the channels must desensitize. The effects of okadaic acid strongly suggest that this results from a dephosphorylation process. The phosphatase 1 and 2a inhibitor enhanced Ca influx during this period and the enhancement was completely blocked by 10 μM MK-801. Thus, there is NMDA-sensitive influx during the second 2.5 min of ischemia if dephosphorylation is prevented. The net dephosphorylation probably results from the fall in ATP during ischemia (MacDonald et al., 1989). The inactivation of the NMDA channel certainly reduces Ca entry during ischemia, and so tends to protect against ischemic damage.

Non-NMDA receptor–mediated influx

About 25% of the increased influx during the second 2.5 min of ischemia was blocked by 50 μM nifedipine, a concentration well above the K_i for this voltage-dependent calcium channel blocker (Godfraind et al., 1986). This might well represent Ca entry through L-type channels, which appear to be clustered at proximal dendrites and somatic regions of the CA1 pyramidal cells (Westenbroek et al., 1990), and which are blocked by 5–10 μM nifedipine in hippocampal slices (O'Dell and Alger, 1991); there is very little nifedipine-sensitive influx of Ca into presynaptic terminals (Carvalho et al., 1986; Suszkiw et al., 1989). Other voltage-dependent Ca and Na channels may also be involved (Pauwels et al., 1990; Richard et al., 1991). Although nifedipine has other, nonchannel, effects at these concentrations, they should not account for the reduced accumulation. The best-established of these effects are inhibition of the Ca-ATPase (Hata et al., 1988) and inhibition of mitochondrial Na/Ca exchange (Vaghy et al., 1982), both of which would increase Ca accumulation. It has been suggested that nifedipine partially blocks plasmalemma Na/Ca exchange (Carvalho et al., 1986), which could account for the inhibition of influx, but in well-controlled studies on heart sarcolemma the drug does not affect this process (Hata et al., 1988). Thus, it seems quite probable that a portion of the Ca uptake is through voltage-dependent Ca channels. Nimodipine blocks part of the increase in cytosolic Ca during focal ischemia, indicating there is a similar pathway *in situ* (Uematsu et al., 1991).

Relationship of calcium fluxes to ischemic damage

The extent of LTF was dependent on tissue Ca levels and was maximal in buffer containing 1.2 mM Ca (Fig. 3). Tissue calcium levels at the end of the 5 min ischemic period in this buffer were identical to those in 2.4 mM Ca buffer prior to the ischemia. Thus, it is not surprising that blocking the ischemic influx in 2.4 mM Ca buffer with a variety of agents failed to protect against damage: the total cell calcium that was present prior to ischemia should have been adequate to cause complete damage. In contrast to this situation, blockade of NMDA-mediated Ca influx by MK-801 did strongly attenuate LTF when slices were in 1.2 mM Ca buffer. In this case, where normal resting cell Ca levels are below those required to cause complete LTF, reducing resting influx and preventing the early influx of Ca with MK-801 was protective. The increased influx during the second 2.5 min was apparently not adequate to cause measurable damage during this short ischemic exposure.

The ability of NMDA antagonists to protect against ischemic damage varies widely in different systems (Simon et al., 1984; Weiss et al., 1986; Clark and Rothman, 1987; Kochhar et al., 1988; Fleischer et al., 1989; Kass et al., 1989; Bullock et al., 1990; Buchan et al., 1991). In the severe ischemic conditions of the present experiments no protection was provided at 2.4 mM extracellular Ca. At 1.2 mM extracellular Ca there was good protection. These varied results can all be explained by the observations that total cell Ca is a determinant of cell damage during ischemia and that NMDA antagonists prevent only one component of Ca entry. Whether or not NMDA antagonists are protective in a given instance will thus depend on whether cell Ca levels rise to damaging levels in the absence of activation of NMDA receptors. That, in turn, will depend on severity of the depletion of cell energy levels and the duration of the insult, as well as the presence of other transmitters, which may also increase cytosolic Ca.

Combining an NMDA antagonist with agents that block activation of AMPA/kainate receptors was able to prevent LTF, even at 2.4 mM extracellular Ca. This was seen with the competitive antagonists DNQX and kynurenic acid and also with agents that block glutamate accumulation during ischemia (DTG or high levels of ketamine; Lobner and Lipton, 1990). Activation of AMPA/kainate receptors thus appears able to cause cell damage, but not via activation of Ca influx during *in vitro* ischemia; DNQX had no effect on ischemic Ca influx. It is possible that DNQX, which is only slowly washed out of the tissue, attenuated Ca influx following the return to normoxic conditions and so protected by actions during the postischemic period, as appears to occur *in situ* (Sheardown et al., 1990). It is also possible that AMPA/kainate binding may enhance Na entry, which would in turn increase efflux of Ca from the mitochondria (Nicholls and Scott, 1980) and so increase cytosolic Ca. Alternatively, the AMPA/kainate receptors may enhance damage by a Ca-independent mechanism (Dykens et al., 1987).

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